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CONTRACT NO: DAMD17-91-C-1012

TITLE: MECHANISMS OF DRUG RESISTANCE IN PLASMODIUM FALCIPARUM

PRINCIPAL INVESTIGATOR: Dyann F. Wirth, Ph.D.

CONTRACTING ORGANIZATION: Harvard University
Department of Tropical Public Health
Harvard School of Public Health
1350 Massachusetts Avenue
Cambridge, Massachusetts 02138

REPORT DATE: June 14, 1994

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. Army Medical Research, Development, Acquisition and Logistics Command (Provisional), Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 14, 1994	Final Report (2/15/91 - 5/14/94)

4. TITLE AND SUBTITLE
Mechanisms of Drug Resistance in Plasmodium Falciparum

5. FUNDING NUMBERS
Contract No.
DAMD17-91-C-1012

6. AUTHOR(S)
Dyann F. Wirth, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Harvard University
Department of Tropical Public Health
Harvard School of Public Health
1350 Massachusetts Avenue
Cambridge, Massachusetts 02138

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research, Development, Acquisition and Logistics Command (Provisional), Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Malaria continues as a major health threat throughout the tropical world and potential demand for antimalarials is higher than for any other medication yet the world faces a crisis--drug resistance is emerging and spreading faster than drugs are being developed and the flow in the pipeline of new drugs has all but stopped. This represents a particular threat to the US Military. In a short time there may be parts of the world where no effective antimalarial drug is available. The recent emergence of multidrug resistant malaria parasites has intensified this problem. The goal of this work is to use a molecular genetic approach in the investigation of mechanisms of drug resistance and subsequently to use this information in the identification and development of new antimalarial drugs. These studies were initiated based on the observation that one mechanism of drug resistance in *P. falciparum* may be similar to multi-drug resistance in cancer. During this work, we identified and fully characterized two mdr-like genes in *P. falciparum*, *pfmdr1* and *pfmdr2* and have found an association with the amplification and overexpression of one of these genes, *pfmdr1* with mefloquine resistance and multidrug resistant parasites both in laboratory derived and field isolated strains of *Plasmodium falciparum*. As a next step in this work, we have initiated the development methods of functional analysis which are critical both to developing and testing new chemotherapeutic interventions.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Malaria, Drug Resistance, Recombinant DNA, Tropical Disease, Infectious Disease	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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5. INTRODUCTION

Malaria continues as a major health threat throughout the tropical world and potential demand for antimalarials is higher than for any other medication yet the world faces a crisis-drug resistance is emerging and spreading faster than drugs are being developed and the flow in the pipeline of new drugs has all but stopped. This represents a particular threat to the US Military. In a short time there may be parts of the world where no effective antimalarial drug is available. The recent emergence of multidrug resistant malaria parasites has intensified this problem. Recognizing this emerging crisis, it is necessary to identify new strategies for the identification and development of new antimalarials. The goal of this work is the development of a framework for antimalarial drug development into the 21st century.

A new strategy for drug development is urgently needed. Current drugs are based on a small number of target molecules or lead compounds and in most cases the target of drug action is yet to be identified. Resistance is emerging rapidly and the mechanisms of resistance are poorly understood. The identification of new targets or new candidate drugs based on an understanding of the parasite biology are key elements in this new strategy. Clearly the development of a new antimalarial will require both basic and applied research working in concert with one another.

The goal of this work is to use a molecular genetic approach both in the identification of new drug targets and in the investigation of mechanisms of drug resistance. These approaches are based on the functional analysis of malaria genes with the goal of using this information in the identification and development of new antimalarial drugs. This is a new strategy and it is being applied because of the crisis facing us in antimalarial drugs. The previous strategy, namely lead directed screening must be supplemented by new strategies or we will be faced with multiresistant *Plasmodium falciparum* and no drugs to treat it.

Importance of drug resistance

Drug resistance has emerged as a major problem in the treatment of all microbial agents and in many cancer chemotherapies. This has necessitated the continuous development of new chemotherapeutic agents both for treatment of infectious agents and for cancer chemotherapy. Often resistance develops through selection of a mutation in the target enzyme of the drug or in the overexpression of that enzyme. For example, resistance to antifolate drugs is frequently associated with mutations in the dihydrofolate reductase enzyme or in its overexpression (Bzik *et al.* 1987, Peterson *et al.* 1990). An alternative type of resistance, namely multidrug resistance has emerged as a major problem in the treatment of many cancers and remains a major obstacle to the successful control of certain neoplasias with chemotherapy. This type of resistance is characterized by several unique features and the molecular basis for this resistance is under extensive investigation (Choi *et al.* 1991, Gros *et al.* 1986, Roninson *et al.* 1986, Raymond *et al.* 1990, Udea *et al.* 1987a, 1987b, Guild *et al.* 1988). In the case of multidrug resistance, resistance is observed to a number of structurally distinct drugs each with a different target. Selection of cells resistant to one drug results in the cross resistance to several structurally and functionally unique drugs. The genes associated with this resistance are the multidrug resistance *mdr* genes. The *mdr* genes encode membrane glycoproteins, the P-glycoproteins which mediate the efflux of drugs from the cell. Amplification of the *mdr*1 gene in resistant cells results in increased expression of the P-glycoprotein and thus increased efflux of drugs. Thus, the cells are resistant because drug is rapidly removed from the cell before significant toxicity occurs. Use of transfection of the *mdr*1 cDNA has demonstrated that overexpression of this gene is sufficient to confer the multidrug resistance phenotype. Drug resistance can be modulated by the use of several compounds including verapamil which appear to inhibit drug efflux. The current hypothesis is that verapamil and related compounds directly bind the P-glycoprotein molecule and block efflux and evidence for direct binding of radiolabelled verapamil to the P-glycoprotein molecule support this hypothesis.

In the case of malaria, the similarity in the pharmacological features of the chloroquine resistance in *P. falciparum*, namely the proposed efflux mechanism and the reversal of resistance by verapamil, desipramine and related compounds led to the proposal that a similar mechanism for drug resistance was operating in *P. falciparum* (Martin *et al.* 1987, Krogstad *et al.* 1992). Both our group under the support of this grant (Wilson *et al.* 1989, Foote *et al.* 1989) and David Kemp's group identified genes that had sequence and predicted structural similarity to the *mdr* genes and have completed the sequence, analyzed the expressed mRNA and protein. These genes and their encoded proteins are indeed related to the family genes in the ATP-Binding Cassette family and have the highest homology with the *mdr* genes from mouse and human. Thus, the hypothesis was proposed that these genes are involved in drug resistance in *P. falciparum*. Further evidence for this proposal was presented by the Foote *et al.* (1990) in identifying several polymorphism within the *Pfmdr*1 gene which appeared to be associated with chloroquine resistance in field isolates. This evidence was in contrast to the analysis by Wellems *et al.* in performing a genetic cross between a chloroquine resistant and chloroquine sensitive cloned parasite (Wellems *et al.* 1990, 1991). In the genetic analysis, both the *pfmdr*1 gene and its assorted polymorphism could be dissociated from chloroquine resistance. This was confirmed by a collaboration between our group and the NIH group in which we sequenced the relevant regions of the polymorphism from the resulting progeny (Wilson *et al.* 1993). Further evidence to refute the association of polymorphism in the *pfmdr*1 gene associated with chloroquine resistance was obtained by sequencing recent isolates of drug resistant *P. falciparum* (Wilson *et al.* 1993). We have completed this and have demonstrated in 12 new isolates of chloroquine resistant parasites, that the *pfmdr*1 gene sequence is identical to that predicted for the chloroquine sensitive phenotype, thus refuting the original Foote et al claim. Further RFLP analysis of the genetic cross by the Wellems group has determined linkage of the resistant phenotype to a small region of chromosome 7, a location distinct from the known location of either *pfmdr*1 or *pfmdr*2. Thus, the conclusion from this work is that neither the *pfmdr*1 or *pfmdr*2 gene is linked to chloroquine resistance.

The mechanisms of chloroquine resistance remains unknown but progress has recently been reported on a putative target for chloroquine drug action. Slater *et al.* (1992) have reported an enzyme activity, heme synthetase which is hypothesized to be involved in the formation of hemozoan pigment and is a method for detoxification of the heme. This enzyme activity in cell extracts is inhibited by chloroquine and related quinones. Interestingly, the enzyme activity is equally sensitive to chloroquine whether derived from chloroquine sensitive or chloroquine resistant parasites. These results indicate progress towards identifying the primary target of chloroquine action and are consistent with the hypothesized importance of efflux of the chloroquine in drug resistance. The increased efflux phenotype remains associated with chloroquine resistance both in the genetic cross experiments and in new chloroquine resistant field isolates (Krogstadt *et al.* 1992, Wellems *et al.* 1990 and Watt *et al.* 1990). In addition reversal of chloroquine resistance with verapamil is observed in all chloroquine resistant strains tested. Thus, the pharmacology of this system remains consistent and has many similarities to the efflux mediated multidrug resistance in mammalian cells. However, the genetic evidence argues strongly that the identified *pfmdr*1 and *pfmdr*2 genes are not linked to the chloroquine resistance phenotype.

The role of *pfmdr* genes in other drug resistance mechanisms remains an open and important question. This is particularly the case for mefloquine resistance in Southeast Asia (Oduola *et al.* 1987, 1988, Nosten *et al*. 1991, Kyle *et al*. 1990, Shanks *et al. 1991).* In our original work, we demonstrated that in a laboratory selected mefloquine resistant cloned parasite, W2mef, the *pfmdr*1 gene was amplified when compared to the cloned parent parasite, W2. In subsequent work, Peel et al have demonstrated that under increased mefloquine selection pressure that the *pfmdr*1 gene is further amplified approximately 8-10 fold. We also demonstrated an increased expression of *pfmdr*1 mRNA in W2mef compared to W2. This work has now been expanded to include several field isolated of mefloquine resistant parasites and our data suggests that in mefloquine resistant parasites in Southeast Asia, an amplification of the *pfmdr*1 gene and an

increased expression of mRNA is associated with this resistance (Volkman *et al.* 1992). Further, analysis of the mefloquine resistant strains from Southeast Asia demonstrates that they are cross-resistant, in vitro, to other unrelated drugs, similar to the cross-resistance observed in multidrug resistant mammalian cells (Wilson *et al.* 1993). Resistance to all drugs can be reversed by penfluoridol and other reversal compounds. Thus, it appears that mefloquine resistant *P. falciparum* has many of the characteristics in common with multidrug resistant mammalian cells, however, definitive proof of this relationship awaits functional analysis.

The protein encoded by the *pfmdr*1 gene has been identified both by our group (Serano *et al.* 1994) and by Cowman and coworkers (1991) using antibody raised against fusion proteins. The P-glycoprotein molecule is 160,000 -170,000 MW and is found associated with membranes in fractionation studies. Cowman finds an association of the protein with the parasite food vacuole and proposes that it is involved with transport in and out of that vacuole. Further investigation of its localization throughout the parasite life cycle and in drug resistant verses drug sensitive parasites is necessary.

While this work was ongoing in *P. falciparum*, both our group and other groups undertook to investigate the potential role of *mdr*-like genes in drug resistance in other parasitic organisms. A similar approach was taken in the identification and cloning of *mdr*-like genes in other parasites. With the collaboration of Dr. Esther Orozco, we cloned two *mdr*-like genes from *Entamoeba histolytica* and demonstrated an association of overexpression of one of these genes with emetine resistance (Samuelson *et al.* 1990 and Ayala *et al.* 1990). This work is being continued by Dr. Orozco and Dr. John Samuelson, who was a postdoctoral fellow and is now an independent investigator in the department.

We also initiated studies of the *mdr*-like genes in *Leishmania enriettii* using the same approach of cloning homologous genes using PCR and primers based on the conserved ATP binding sites of the *mdr* gene family, and cloned several independent *mdr*-like genes. In collaboration with Dr. Buddy Ullman, Oregon Health Science University, we have identified one of these genes, *ldmdr1*, as amplified in vinblastine resistant *L. donovani* (Henderson *et al* 1992). In parallel work in *Leishmania tarentole*, Oulette and Borst identified five *mdr*-like genes, one of which was located on the H-region resistance element (Oulette *et al.* 1991, 1991a, 1991b). Subsequent work by Callahan and Beverley (1991) and Oulette *et al.* (1991) have demonstrated this *mdr*-like gene is associated with arsenite resistance in these parasites. The gene identified as associated with arsenite resistance is distinct based on DNA sequence and chromosome location from *ldmdr1* which is associated with vinblastine resistance. Thus, in Leishmania, there are at least five *mdr*-like genes, two of which are associated with drug resistance. In the case of arsenite resistance, the pgpa gene has been transfected into a sensitive strain of parasite and there has been a clear demonstration of the association of this gene with arsenite resistance (Callahan and Beverley *et al.* 1991). We have tested both the *ldrmdr1* gene and its homologue cloned from *Leishmania enriettii*, the *lemdr1* gene, by cloning in the expression vector pALTNEO and transfection into wild type *L. enriettii*. The resulting transfected cells were resistant to vinblastine, thus demonstrating the role of the *lemdr1* and *ldmdr1* genes in drug resistance (Chow et al, 1993, Henderson et al 1992).

The observation that amplification and increased expression of *mdr*-like genes in *Leishmania sp.* is associated with drug resistance in these parasites provides an excellent model system for the analysis of the detailed mechanism of this resistance in a parasitic protozoal system and should provide insights into the analysis of drug resistance in these organisms. The advantage to the Leishmania system is the ability to transfect the parasite (Laban *et al.* 1990 and Kapler *et al.* 1990) and thus site specific mutagenic analysis of the *mdr*-like genes can be readily undertaken. This has been an extremely powerful approach in mammalian cell systems in the analysis of the *mdr* genes and their functional domains.

It is interesting to note that there are at least five genes in Leishmania. This is in contrast to mammalian cells in which there are three genes, two of which are closely related. Thus, one immediate result of the work in Leishmania is that we are pursuing potential additional *mdr*-like genes in *P. falciparum* with the notion that although chloroquine resistance in not linked to the two known genes, there may be additional, unidentified *mdr*-like genes, one of which may be involved in chloroquine resistance.

6. BODY

Progress has been made on all of the original technical objectives and summaries of the work are included in this section and detailed descriptions in the accompanying appendix material. In addition, recent progress has been made in developing a homologous transfection system for the malaria parasite. This is the first step in the development of a system for the functional analysis of various parasite genes both in the investigation of the mechanisms of drug resistance and in the identification and testing of new targets for drug development.

Identification of mdr genes in *P. falciparum*

These studies were initiated based on the observation that one mechanism of drug resistance in *P. falciparum* may be similar to multi-drug resistance in mammalian cells, namely the mediation of drug efflux by an ATP-dependent efflux pump. A prediction of this model was that the parasite would have *mdr*-like genes and that these would be involved in drug resistance. We used sequences that are conserved in the mammalian P-glycoproteins and several bacterial transport proteins to identify putative *mdr*-like genes in *P. falciparum*. Two primers based on conserved protein sequences shared in the mouse *mdr*, human *mdr* and the bacterial hemolysin B(HlyB) proteins were synthesized. The sense primer was based on a nine amino acid homology found in position 1066-1075 and the antisense primer was based on a seven amino acid homology found in position 1198 to 1204 of the murine *mdr* gene. The codon usage was based on the preferred codon usage for *P. falciparum*.

Two *mdr*-like genes were identified in *P. falciparum* using this approach, *pfmdr*1 and *pfmdr*2. *pfmdr*1 was independently identified by the David Kemp's group. Most of our research effort has been focused on the *pfmdr*1 gene because of its association with mefloquine resistance in *P. falciparum* (see below). We have sequenced the entire *pfmdr*1 open reading frame from a number of independent *P. falciparum* strains (see below) and the gene encodes a protein with strong homology with mammalian *mdr* genes both in primary sequence in the highly conserved putative ATP-binding regions and with predicted structural similarity in the transmembrane regions. By Northern analysis (see Volkman *et al.* 1993), we have demonstrated that the *pfmdr*1 gene is expressed at all stages of the asexual parasite life cycle, but that there are different mRNA sizes in rings (single 8.5 kb mRNA) and trophozoite (8.5 kb and 7.5 kb mRNAs). These mRNAs are larger than would be predicted based on the size of the open reading frame (4.5 kb) and characterization of the structure of these mRNAs is underway through a combination of reverse transcriptase-PCR analysis, primer extension analysis and nuclease protection analysis. Reverse transcriptase-PCR analysis demonstrates that across the coding region of the *pfmdr*1 gene both mRNAs are colinear with the genomic DNA. In the region 3' to the end of the coding region, there is a divergence between the genomic and mRNA sequence as demonstrated and the basis of this difference is currently being investigated by direct sequence analysis of the PCR products derived from genomic DNA and mRNA.

Association of pfmdr1 amplification with mefloquine resistance

Our analysis of *mdr* genes and drug resistance began with a study of the cloned Indochina strain W2 and its derivative, W2mef which was selected in in vitro culture with increasing concentrations of mefloquine. When we analyzed the *pfmdr*1 gene and its expression in these parasites, we discovered that the *pfmdr*1 gene was amplified 4 fold and there was an increased expression of the *pfmdr*1 mRNA. There was no apparent change either in gene copy number or expression of *pfmdr*2 gene.

We wanted to determine if the amplification of the *pfmdr*1 gene and the increased expression of its mRNA were general properties of mefloquine resistant *P. falciparum*. We therefore extended these studies to mefloquine resistant parasites isolated from patients who failed mefloquine treatment in Eastern Thailand in collaboration with Dr. S. Thaithong. Each parasite strain was tested in vitro for drug sensitivity and then analyzed for *pfmdr*1 gene copy number and expression.

The results of sensitivity testing are shown in Wilson *et al.* 1993 (See Appendix). All of the isolates were tested for sensitivity to chloroquine (CLQ), mefloquine (MFQ), quinine (QUIN), desythylchloroquine (DCQ), halofantrine (HAL), and artemisinin (QHS). Isolates PR145, TM327, TM336,TM338,TM342,TM343,TM345,TM346,TM347,TM352 all exhibit a decreased sensitivity to mefloquine when compared to a mefloquine sensitive strain, TM335, isolated from the same geographic region. Interestingly, those parasites resistant to mefloquine show a decreased sensitivity to halofantrin, another aminoquinoline, which has not been used in Thailand. These in vitro results are in agreement with recent reports of clinical resistance of *P. falciparum* to halofantrin in Eastern Thailand. Additional work needs to be done in order to determine if this is indeed multi-drug resistance occurring in response to treatment with mefloquine.

Amplification and expression of the *pfmdr1* gene in mefloquine resistant parasites.

The gene copy number was determined by quantitative Southern analysis for each of the mefloquine resistant isolates from Thailand. The CSP gene was used as an internal control for each experiment. To confirm that the Thai isolates were distinct, the isolates were analyzed using a repetitive probe which gave a unique fingerprint for each strain. The gene copy number results represent compiled data from three separate experiments. In all of the mefloquine resistant Thai isolates tested, the *pfmdr*1 gene is amplified, while in the mefloquine sensitive isolate from the same geographic area, there is a single gene copy. These results are consistent with those we originally described using W2 and its mefloquine resistant daughter, W2mef, namely, in mefloquine resistant parasites, there is an increased copy number of the *pfmdr*1 gene.

We next examined expression of the *pfmdr*1 gene in a subset of the mefloquine resistant parasites by quantitative Northern and RNA dot blot analysis. There is an increased expression of *pfmdr*1 mRNA in mefloquine resistant parasites, either isolated from the field or derived in the laboratory (see Volkman *et al.* 1993 and Wilson *et al.* 1993 in the Appendix)

Pfmdr1 sequence

While this work as ongoing, the Kemp group reported an association of certain polymorphism in the predicted amino acid sequence of the *pfmdr*1 gene product with chloroquine resistance. Thus, we were interested in determining if a set of polymorphism in the *pfmdr*1 gene product could be associated with mefloquine resistance. We sequenced the *Pfmdr*-1 gene in isolates TM352, TM346 and TM335 to explore the possibility of specific sequences being associated with the drug resistance pattern of these isolates as had been previously suggested for chloroquine. We found a somewhat surprising result, namely the only difference from the original

sequence was at bp 1051 (amino acid 184, TAT to TTT, Tyr to Phe). This sequence was shared by all three genes, two from mefloquine resistant parasites TM352 and TM346 and one from a mefloquine sensitive parasite, TM335. Therefore, we concluded that there was no association of this change with mefloquine resistance. However, in comparing our sequence of the these genes to those reported by the Kemp group, we noticed that all of our parasite strains are resistant to chloroquine and yet none of them had the sequence which Kemp's group had predicted as associated with chloroquine resistance. We therefore sequenced the regions of predicted polymorphism in the remaining Thai isolates and found them to be identical to those isolates in which we had sequenced the full-length gene. Thus, despite the chloroquine resistant phenotype of these parasites (which we confirmed by our own in vitro testing), they did not contain any of the polymorphism identified by the Kemp group as being associated with chloroquine resistance. This result is consistent with the recent work of Wellems which failed to find genetic linkage of *pfmdr*1 with chloroquine resistance.

Characterization of *pfmdr2* gene

We have now completed our characterization of the *pfmdr* 2 gene and this work is published (See Zalis *et al.* 1993 in Appendix). Briefly, our major findings are that this gene is related to the P-glycoprotein family but has a somewhat different structure than the *mdr* genes. Based on DNA sequence analysis of genomic clones, we have discovered that the *pfmdr*2 gene has 10 predicted transmembrane domains and a single ATP-binding site at the 3' end of the gene. In a homology search using GenBank sequences, we discovered that the *pfmdr*2 gene has a significant homology with the *hmt1* gene in yeast. The yeast *hmt1* gene is involved in cadmium resistance and is hypothesized to transport cadmium containing complexes from the cell. The predicted structure of the *hmt1* gene is identical to that of *pfmdr*2, namely 10 transmembrane domains and a single ATP binding site. We have further characterized the *pfmdr*2 gene expression by northern analysis and discovered that it is expressed in a stage specific manner, only at the trophozoite stage and not in ring stages. We have prepared a rabbit antibody to a recombinant fusion protein expressing a portion of the *pfmdr*2 coding region. In IFA analysis by confocal microscopy, this antibody stains trophozoites and not ring stages and the antibody staining appears to have a vacuolar localization. Western analysis reveals a protein of approximately 110 kd which is consistent with the size of the predicted open reading frame based on DNA sequence analysis. Based on this analysis and previous work, there is no evidence for a change in *pfmdr*2 expression in drug resistant verses drug sensitive parasites. However, based the similarity with the *hmt1* gene of yeast, we plan to investigate the role of this gene in heavy metal resistance in the parasite.

Transfection of the malaria parasite and transient expression of firefly luciferase

Malaria remains a major threat to world health. Efforts to control the disease have focused on chemotherapy, mosquito control and most recently vaccine development. These efforts have been hampered by the emergence and spread of drug resistant parasites, the breakdown of malaria control programs both due to insecticide resistant mosquitoes and upheavals in spraying programs and the complicated problems of vaccine development and testing. The world is facing an increasing threat of malaria in the 1990s and with few new tools to combat the parasite and disease.

One of the underlying problems in developing newer methods of control is that the basic biology of the parasite has not been fully investigated, primarily due to the lack of a method for functional analysis of genes and their products. The goal of the work presented here is to develop a method for the functional analysis of genes using the method of DNA transfection. This type of methodology is a critical next step in the functional analysis of parasite genes and is required for a detailed analysis of the control of expression of parasite genes. Such methods have been critical in dissecting the mechanisms of bacterial pathogenesis and in the development of vaccines for several important bacterial pathogens.

The malaria parasite presents a unique challenge for transfection in that it is intracellular in most of its life cycle and thus introduced DNA must cross multiple membrane barriers before reaching the parasite nucleus. Because these multiple barriers would be likely to reduce the efficiency of introducing DNA into the parasite we chose to use a parasite stage which was extracellular, this being the female gamete and fertilized zygote. Methods had previously been developed for the purification of gametes and fertilized zygotes of the avian malaria *Plasmodium gallinaceum*. Further, several genes of *P. gallinaceum* have been identified which are expressed at high level in the gamete/zygote stages of the parasite and one of these, pgs28 had been cloned with adequate flanking DNA to assume that necessary 5' and 3' controlling elements for the expression of this gene. We now report the development of a transient transfection vector by constructing a chimeric gene in which the firefly luciferase gene was inserted in a frame into the coding region of the pgs28 gene of *Plasmodium gallinaceum*. This plasmid DNA was introduced into *P. gallinaceum* gametes and fertilized zygotes by electroporation and luciferase expression was assayed after 24 hours. This is the first demonstration of successful introduction and expression of a foreign gene in the malaria parasite and demonstrates the feasibility of this approach to developing methods for the functional analysis of parasite genes (see Goonewardene *et al* 1993 in the Appendix).

This represents a significant departure in methodology from the previous work. This is because of a recent breakthrough we have made in the development of a homologous transfection system for the malaria parasite. Much of the work now focuses on the characterization and further development of that system. Once the methodology is in place, we will use the homologous transfection system to test the role of *mdr*-like genes in drug resistance. Based on our experience in transfection of Leishmania species and the important role of functional testing of genes by methods of overexpression and targeted gene knockout, I feel that the most efficient and scientifically valid approach is the study these genes in a homologous transfection system. As is described, we have recently been able to develop a transient transfection system using the sexual stages of *Plasmodium gallinaceum* as a model system and we now propose to continue this work both in the characterization of that system, the development of a stable selectable marker, the transfection of asexual stage parasites and finally the development or transfer of this system to the human malaria parasite, *Plasmodium falciparum* . Some of these experiments will be attempted in parallel and clearly with this roadblock eliminated, I anticipate significant activity in many laboratories such that some of these methodologies may become available relatively rapidly. Once the system is in place, we will test the role of the *pfmdr1* and *pfmdr2* genes in drug resistance using the methodologies developed under this grant. Our focus in this work will remain the genetic and molecular basis of drug resistance in *Plasmodium falciparum* and the experiments described are directed toward developing methodologies necessary to answer those questions. In addition, the development of this methodology will have many spinoffs including the development of genetically altered strains of the malaria parasite which may be useful in vaccine development and testing.

7. Conclusions and future experiments

Our results implicate a role for the amplification of the pf*mdr*1 gene in mefloquine resistance in *P. falciparum*, however, the results remain correlative rather than functional. Our work will focus primarily on functional analysis of *mdr*-like genes in parasitic protozoa and we will attempt to develop methods to functionally test the *P. falciparum mdr*-like genes, including expression in heterologous systems and development of an endogenous transfection system. In my scientific judgement, the further development of a transfection system is essential for future work in functional analysis of the pf*mdr* genes and has the added advantage of being useful in many other applications and should have the highest priority.

The overall goal of this work is to understand the role of *mdr*-like genes in drug resistance and to use this information in the development of new drugs for malaria. *Mdr*-like genes have

been identified in several organisms and there is an association of overexpression of these genes with a multidrug resistant phenotype. This type of multi-drug resistance phenotype represents a threat to any new drug and thus a fundamental understanding of its mechanism and the development of drugs to reverse this resistance are of the highest priority.

The *Plasmodium falciparum* gene will be tested in heterologous systems and significant effort will be devoted to developing a homologous transfection system for *P. falciparum*.. Progress has been made as reported above in the development of a transient transfection system for *Plasmodium gallinaceum* and every effort to transfer this technology to the *Plasmodium falciparum* system is being made. While this represents a high risk aspect of the project, the value of such a system for the analysis of drug resistance mechanisms and for the analysis of several other important features of parasite biology, it is a worthwhile undertaking. We have significant expertise in developing transfection systems and have experience in the biology of the parasite. There are other laboratories both in the U.S. and elsewhere who are attempting to develop transfection systems for *P. falciparum*, each with a somewhat different approach. Such multiple efforts greatly enhanced the progress in the field of kinetoplastidae transfection and should one of the other groups be successful, we will use that methodology to investigate the role of *mdr*-like genes in drug resistance in *P. falciparum*. Our interest is in understanding these genes and their function and the development of a transfection system will be an important component in that analysis.

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(9) APPENDIX

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 / 0166-6851/93/$06.00

MOLBIO 01875

Amplification of *pfmdr1* associated with mefloquine and halofantrine resistance in *Plasmodium falciparum* from Thailand

Craig M. Wilson[a]*, Sarah K. Volkman[a], Sodsri Thaithong[b], Roger K. Martin[c], Dennis E. Kyle[c], Wilbur K. Milhous[c] and Dyann F. Wirth[a]

[a]*Harvard School of Public Health, Boston, MA, USA;* [b]*WHO Collaborating Centre, Chulalongkorn University, Bangkok, Thailand; and* [c]*Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, DC, USA*

(Received 29 June 1992; accepted 8 September 1992)

Drug resistance in *Plasmodium falciparum* is an expanding problem in most endemic areas. Recent studies have suggested the potential involvement of genes in the MDR gene family in resistance to quinoline-containing compounds in *P. falciparum*. In this study a molecular analysis of *pfmdr1* in recent isolates from Thailand was done (1) to further examine the role of *pfmdr1* in drug-resistant isolates and (2) to examine the reported association of *pfmdr1* intragenic alleles and chloroquine resistance. Most of the isolates (10 of 11) were resistant to all compounds tested. Analysis of *pfmdr1* revealed an apparent association between increased gene copy number and increased level of expression of *pfmdr1* and decreased susceptibility to mefloquine and halofantrine. Sequence analysis of *pfmdr1* in these isolates revealed no association of intragenic alleles with chloroquine resistance.

Key words: Malaria; *Plasmodium falciparum*; Drug resistance; Multiple drug-resistant; Mefloquine; Halofantrine

Introduction

Malaria remains one of the major world public health problems. Drug-resistant strains of *Plasmodium falciparum* are increasingly prevalent in most endemic areas complicating treatment, control and prophylaxis and rendering even newer therapeutic modalities rapidly useless [1]. Due to the rapid and predictable emergence of resistance to the antifolate antimalarials in *P. falciparum*, management of these infections is increasingly dependent on quinoline-containing compounds.

Previous studies demonstrating the efflux of chloroquine (CLQ) from resistant parasites [2] and modulation of this efflux and resistance by verapamil [3] suggest a role for a gene of the multiple-drug resistance (MDR) family in chloroquine resistance in *P. falciparum*. Two homologues of the MDR gene family which are part of the ATP-binding cassette (ABC) gene superfamily [4] have been identified in *P. falciparum* based on sequence and predicted structural homology [5,6]. Despite the prediction for the involvement of such genes in CLQ resistance, the role of these homologues in CLQ resistance remains unsubstantiated. One of the homologues, *pfmdr1*, is amplified in some but certainly not all CLQ resistant strains [6,7]. Analysis of allelic variations in the *pfmdr1* gene along with a 3′ polymorphism revealed an apparent association with CLQ

Correspondence address: Dyann F. Wirth, Department of Tropical Public Health, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115, USA. Tel: (617) 432-1563 Fax: (617) 738-4914.

**Present address:* Division of Geographic Medicine, University of Alabama at Birmingham, Birmingham, AL, USA.

Abbreviations: CLQ, chloroquine; MFQ, mefloquine; HAL, halofantrine; QHS, artemisinin; DCQ, desethylchloroquine; MDR, multiple-drug resistance; ABC, ATP binding cassette; PCR, polymerase chain reaction.

resistance [8]. However, analysis of the progeny of a cross between a CLQ resistant and a CLQ sensitive clone showed that the parental *pfmdr1* genotype could be dissociated from the resistance and reversal phenotypes [9], arguing that *pfmdr1* is not involved in CLQ resistance. This conclusion is further supported by the genetic mapping of a chloroquine-resistant locus to chromosome 7 using the same cross [10]. There is no apparent role for *pfmdr2* in CLQ resistance [5,9].

Mefloquine (MFQ) resistance has other characteristics of the MDR phenotype. The acquisition of MFQ resistance is often concurrent with decreased susceptibility to halofantrine (HAL) and structurally dissimilar compounds [11, 25]. MFQ, HAL and artemisinin (QHS) $IC_{50's}$ can all be modulated in vitro by penfluridol but not generally by compounds which modulate resistance to the chloroquine-related compounds (DEK unpublished data).

The purpose of these studies was to further examine the potential role of *pfmdr1* in resistance to quinoline-containing compounds in *P. falciparum*. Isolates from patients failing conventional therapies in Thailand were first characterized for drug sensitivity. The gene copy number, sequence and mRNA expression of *pfmdr1* was examined in all of the isolates. There is an apparent association between concurrent acquisition of decreased sensitivity to MFQ and HAL and an increase in gene copy number and gene expression of *pfmdr1*.

A separate purpose of these studies was to examine the sequence of *pfmdr1* in these isolates for evidence of an association of intragenic 'alleles' and drug resistance as had previously been suggested for CLQ resistance [8]. The full-length sequence of *pfmdr1* was determined in selected isolates and the intragenic alleles and 3' polymorphism previously associated with CLQ resistance [8] were determined in all of the islolates. In contrast to the previous report, the sequence analysis of *pfmdr1* in these isolates revealed no association of intragenic alleles and CLQ resistance. In addition the patterns of 3' polymorphism were quite diverse as compared to that previously described [8].

Materials and Methods

Parasites All parasites were maintained in vitro according to a modification of the method of Trager and Jensen [12]. The isolates were maintained in a 5% suspension of type A+ erythrocytes in RPMI 1640 supplemented with 32 mM $NaHCO_3$, 12 mM TES, 37 mM hypoxanthine, 2 mM glutamine, 10 mM glucose, and 10% human plasma in an atmosphere of 1% O_2/ 5% CO_2, balance nitrogen.

The 11 clinical isolates used in this study were isolated from patients reporting to the Tropical Medicine Hospital (TM prefix) or Pong Nam Ron, Chantabori Province (PR prefix) in 1990. The isolates were stabilized in vitro and transported in culture for further testing. The GH2 isolate and its clone GA3 were isolated in 1989 from a patient in the Chantaburi Province area.

The W2 and W2mef clones from Indochina [13] were used as controls in the nucleic acid quantitation studies. In addition the HB3 [14] clone of Honduras I/CDC was used in the sequencing analysis. The W2 clone from Indochina and the D6 [15] clone from Sierra Leone I/WRAIR were used as controls for the drug sensitivity assays.

Sensitivity testing Sensitivity testing was done using the incorporation of [G-^{3}H]hypoxanthine according to a modification of the method of Desjardins et al. [16]. The assays were done in 96-well plates with a 1% erythrocyte suspension at 0.2–0.5% parasitemia in RPMI 1640 supplemented with 25 mM Hepes/ 32 mM $NaHCO_3$/ 10% human plasma in a final total volume of 225 μl prior to adding [G-^{3}H]hypoxanthine. Serial dilutions of the drug being tested were done in duplicate for each assay. The incorporation data were analyzed by nonlinear regression to generate an IC_{50} [16].

Sequencing The sequence of *pfmdr1* for all of the isolates was determined using a modification of a method described for direct sequencing of PCR products after generating single-

stranded DNA by λ Exonuclease (Bethesda Research Laboratories, Gaithersburg, MD) digestion [17]. Serial overlapping oligonucleotide primer pairs were synthesized based on our previous sequence analysis of *pfmdr1* from HB3, W2 and W2mef and the published full-length sequence [6]. One of the primer pairs from each set was kinased using T4-polynucleotide kinase and used in a standard PCR reaction (30 pmol of each primer/ 200 μM dNTPs/ 1–200 ng of genomic DNA as template/ 70 mM Tris pH 8.8/ 20 mM NH_4SO_4/ 2 mM $MgCl_2$/ 1 mM DTT/ 10 μg ml^{-1} BSA, 0.1% Triton X-100 in a 100 μl reaction for 25 cycles of 94°C × 1 min, 50°C × 2 min, and 72°C × 3 min). The PCR product was directly extracted with glass beads, resuspended in water (44 μl), adjusted with 5 μl 10 × λ Exonuclease buffer (670 mM glycine-NaOH, pH 9.4/ 25 mM $MgCl_2$/ 500 μg ml^{-1} BSA) and digested with λ Exonuclease (1 μl, about 4 U) at 37°C for 10 min. This product was phenol/chloroform extracted, ethanol precipitated and resuspended for use in a dideoxynucleotide chain termination sequencing reaction using Sequenase^R enzyme. Multiple sequencing primers were used to sequence PCR products of up to 1400 bp.

Southern analysis Genomic DNA was extracted from saponin lysed parasites after Sarkosyl (1%), RNAse A (100 μg ml^{-1}, 1 h at 37°C) and proteinase K (200 μg ml^{-1}, >2 h at 50°C) treatment in the presence of NET buffer (150 mM NaCl/ 10 mM EDTA/ 50 mM Tris pH 7.5). Approximately 1 μg of genomic DNA was digested with *Eco*RI, resolved on a 1% agarose gel, and transferred to a nylon membrane. Hybridization was done simultaneously with a PCR generated fragment of *pfmdr1* (5′ probe: nucleotides 510–1487; 3′ probe:nucleotides 3619–4742) and the CSP gene [18] which had been radiolabeled using a random oligonucleotide priming technique [19]. Hybridization was done in 1% SDS, 500 mM NaCl, 10% PEG, 50% Formamide at 42°C overnight, then washed at a final stringency of 2 × SSC/1% SDS at 55°C for 1 h.

Quantitative analysis of the hybridization signals was done on a Betascan analyzer (Betagen, Waltham, MA). Windows for *pfmdr1* and CSP were counted, background signal was subtracted and ratios of the signal of *pfmdr1* to CSP were calculated for each parasite isolate tested. The ratios were normalized to the ratio obtained for W2 which was known to have a single copy of *pfmdr1* [5].

Isolate characterization Confirming the unique character of the isolates was done using the 3′ polymorphism of the *pfmdr1* gene and by a fingerprinting technique. The PCR based procedure established by Foote et al. [8] was used to look at the 3′ polymorphism of *pfmdr1*. The same oligonucleotide primer pairs and PCR conditions were used. The PCR product was resolved on a 1.8% agarose gel visualized with ethidium bromide and photographed. The predicted PCR product size based on the published sequence [5] would be 860 bp. The multiple bands seen are present in all isolates with variable patterns (ref. 8 and unpublished data CMW).

Fingerprinting of the isolates was performed using a cloned chromosomally-dispersed repetitive element, pC4.H32, as previously described by Dolan et al. [20]. Genomic DNA was digested with *Hin*fI (New England Biolabs, Beverly, MA), resolved on a 1.2% agarose gel, transferred to a nylon membrane and hybridized as above with radiolabeled pC4.H32.

mRNA quantitative analysis RNA was extracted from synchronized parasites as described elsewhere [5]. Multiple samples of RNA were quantitated by dot blot analysis by counting the regions of nitrocellulose directly in a β-scintillation counter. Total nucleic acid was denatured, loaded and hybridized with either a radiolabelled *pfmdr1* or an rDNA probe. Ratios of the counts of *pfmdr1* to rDNA were calculated to adjust for loading. These ratios were normalized to W2, the MFQ sensitive strain.

Results

Sensitivity testing The isolates were first characterized for their drug resistance patterns and the results are shown in Table I. All of the isolates were tested for sensitivity to chloroquine (CLQ), mefloquine (MFQ), quinine (QUIN), desythylchloroquine (DCQ), halofantrine (HAL), and artemisinin (QHS). The numbers shown represent the average results of two separate experiments. All of the isolates were resistant to CLQ, DCQ and QUIN with reduced susceptibility to QHS. Ten of 11 isolates were resistant to MFQ and had reduced susceptibility to HAL; only TM335 was sensitive to MFQ and HAL. Using a

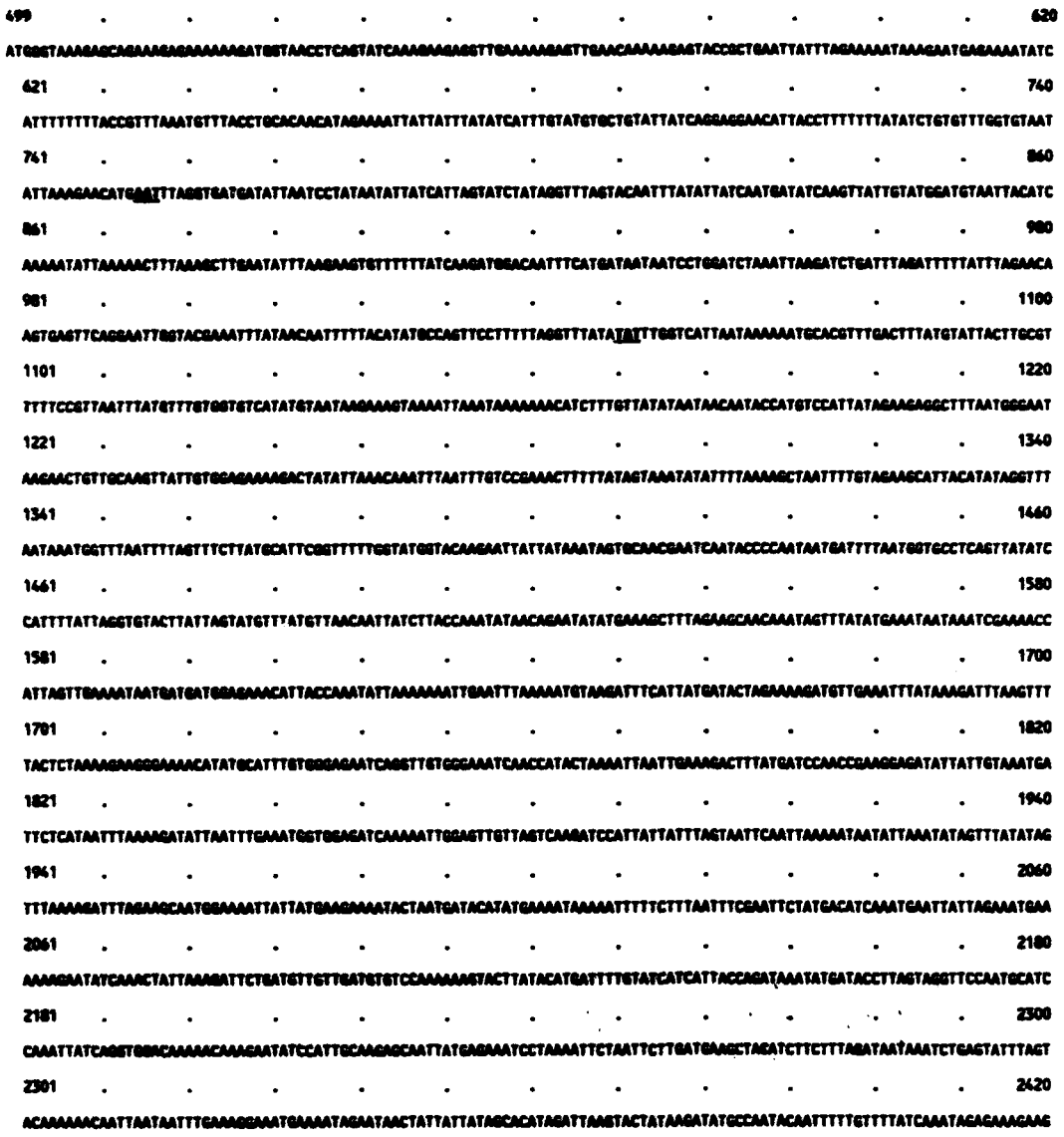

Fig. 1. Sequence of *pfmdr1*. The full-length sequence of *pfmdr1* is given for GA3. The numbering scheme is as used in reference 6. The locations of the polymorphisms which were analyzed are underlined. The sequences of the polyasparaginated region are given for the other three isolates which were sequenced full-length (nucleotides 2425–2475) The data summarizing the sequence analysis is given in Table II.

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3621  .  .  .  .  .  .  .  .  .  .  .  .  .  .  2540
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TGATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAAT...AACAAAATTAATAATGAGGGTAGCTATATTATTGAACAAGGTACACATGATAGTCTTATGAA
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TH535 & TH552 AATAATAATAATAATAATAATAATAATAAT

TH546 AATAATAATAATAATAAT/AAT

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2541  .  .  .  .  .  .  .  .  .  .  .  .  .  .  2660
AAATAAAAATGGTATTTATGATCTTATGATAAATAATCAAAAGTTTCATCAAATAAATCTTCAAATAATGGAAATGATAATGGATCGGATAACAAAAGTAGCGCTTATAAAGACTCAGA
2661  .  .  .  .  .  .  .  .  .  .  .  .  .  .  2780
TACAGGTAATGATGCAGATAATATGAATAGTTTATCAATACATGAAAATGAAAATATATCAAATAATCGTAATTGTAAAAATACAGCAGAAAATGAAAAGAAGAGAAAGTTCCATTTTT
2781  .  .  .  .  .  .  .  .  .  .  .  .  .  .  2900
CAAAAGAATGTTTAGAAGAAAAAAGAAAGCACCAAACAATTTACGTATCATTTATAAAGAAATATTTTCATATAAAAAAGATGTTACTATAATTTTCTTTAGTATTTTAGTAGCTGGAGG
2901  .  .  .  .  .  .  .  .  .  .  .  .  .  .  3020
ATTATATCCCGTATTTGCTTTATTATATGCTAGATATGTATCTACATTATTTGATTTTGCAAATCTAGAATATAACTCAAATAAATATTCTATATATATTCTACTTATTGCTATTGCTAT
3021  .  .  .  .  .  .  .  .  .  .  .  .  .  .  3140
GTTCATTTCAGAAACACTCAAAAACTATTATAACAACAAAATAGGAGAAAAAGTCGAAAAGACTATGAAACGTAGATTATTTGAAAATATATTATATCAAGAAATGAGTTTCTTTGATCA
3141  .  .  .  .  .  .  .  .  .  .  .  .  .  .  3260
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3261  .  .  .  .  .  .  .  .  .  .  .  .  .  .  3380
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3381  .  .  .  .  .  .  .  .  .  .  .  .  .  .  3500
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3501  .  .  .  .  .  .  .  .  .  .  .  .  .  .  3620
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3621  .  .  .  .  .  .  .  .  .  .  .  .  .  .  3740
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3741  .  .  .  .  .  .  .  .  .  .  .  .  .  .  3860
GTCCTTAAAAGGAGATTCAGAAAATGCAAAATTATCATTTGAGAAATATTATCCATTAATGATTAGAAAATCAAATATTGATGTAAGAGATGATGGTGGAATAAGAATAAATAAAAATTT
3861  .  .  .  .  .  .  .  .  .  .  .  .  .  .  3980
AATAAAAGGTAAAGTTGATATTAAAGATGTAAATTTCCGTTATATTTCAAGACCAAATGTACCTATTTATAAAAATTTATCTTTTACATGTGATAGTAAAAAAACTACAGCAATCGTTGG
3981  .  .  .  .  .  .  .  .  .  .  .  .  .  .  4100
AGAAACAGGTAGTGGAAAATCAACTTTTATGAATCTCTTATTAAGATTTTATGACTTGAAAATGATCACATTATATTAAAAAATGATATGACAAATTTTCAAGATTATCAAAATAATAA
4101  .  .  .  .  .  .  .  .  .  .  .  .  .  .  4220
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4221  .  .  .  .  .  .  .  .  .  .  .  .  .  .  4340
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4341  .  .  .  .  .  .  .  .  .  .  .  .  .  .  4460
TAGAACGTGTTAGGAAGTTTGCTGCTATAGATGAATTTATCGAATCATTACCAAATAAATATGATACAAATGTTGGACCATATGGTAAAAGCTTATCAGGTGGACAAAACAGAGAATAGC
4461  .  .  .  .  .  .  .  .  .  .  .  .  .  .  4580
TATAGCTAGAGCATTATTAAGAGAACCTAAAATATTATTATTAGATGAAGCAACATCATCACTTGATTCCAATTCTGAGAAATTAATTGAAAAACTATTGTAGATATTAAAGATAAAGC
4581  .  .  .  .  .  .  .  .  .  .  .  .  .  .  4700
TGACAAAACTATTATTACTATTGCCCACAGAATTGCATCTATAAAACGATCAGACAAAATTGTGGTATTTAATAACCCTGATCGAAATGGAACCTTTGTACAGTCACATGGAACACACGA
4701  .  .  .  .  .
TGAATTATTATCAGCACAAGATGGAATATATAAAAATATGTAAAATTAGCTAAATGA
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separate 72-h morphological assay all the isolates were found to be also resistant to amodiaquine and pyrimethamine (data not shown, S. Thaithong).

pfmdr1 sequence The *pfmdr1* gene was sequenced full length in isolates TM352, TM346, TM335 and GA3 to explore the possibility of intragenic alleles being associated with the drug resistance pattern of these isolates as had been previously suggested for chloroquine [8]. The full sequence of the predicted open reading frame for GA3 is given in Fig. 1 and it matches completely with the previously published sequence. The regions which were sequenced for all of the isolates are underlined. The highly conserved nature of the primary sequence of this gene was once again obvious. As can be seen in Table II, the only nucleotide difference from the published sequence [6] was at bp 1051 (amino acid 184) and was a difference that had been previously noted (TAT to TTT, Tyr to Phe) [8]. This region was sequenced in the remaining isolates along with the other previously identified 'alleles' [8] and the results are given in Table II. The change seen at amino acid position 184 was the only difference noted in all 11 isolates.

There was variation in the number of predicted asparagines noted in the polyasparaginated region of the linker domain (bp 2426 through 2483, Fig 1). As can be seen in Fig. 1, this region contains a predicted run of 7 Asn followed by two aspartic acids and then another series of Asn residues varying in number in different isolates from 5 to 12 (ref. 7 and unpublished data, CMW). The sequence analysis over this region for the isolates which

TABLE I

Drug sensitivity testing

Strain	IC_{50} (ng/ml)					
	CLQ	MFQ	QUIN	DCQ	HAL	QHS
D6	4	15	23	6	1.1	2.3
W2	65	2	74	250	0.3	2.2
TM335	68	6	65	267	0.61	3.0
PR145	35	10	90	168	2.1	2.0
TM327	42	16	59	158	2.7	2.7
TM336	38	15	87	182	2.8	1.6
TM338	29	9	53	170	1.9	1.9
TM342	46	8	80	200	2.1	2.4
TM343	39	14	99	290	3.3	3.5
TM345	65	9	128	62	2.3	3.2
TM346	31	20	114	135	3.7	2.9
TM347	23	17	62	84	3.6	3.1
TM352	27	22	102	117	3.0	3.4
GA3	41	11	49	161	2.0	1.9
Sensitive	<10	<8	<25	<10		

Results are given as IC_{50}s from a standard 48 h hypoxanthine uptake assay. The sensitive levels are those established by WRAIR and reference 1 and 25. HAL and QHS 'sensitive' levels are not clearly established with correlative in vitro and clinical data. CLQ, chloroquine; MFQ, mefloquine; QUIN, quinine; DCQ, desethylchloroquine; HAL, halofantrine; QHS, artemisinin.

TABLE II

pfmdr1 sequence comparison

Amino Acid No.[a]	86	184	1034	1042	1246	Poly-Ns[b]
Nucleotide No.[a]	754	1049	3598	3622	4234	
Isolate						
Sensitive[c]	AAT	TAT	AGT	AAT	GAT	
R:SE Asia[c]	TAT	TAT	AGT	AAT	GAT	
R:S Amer[c]	AAT	(TTT)	TGT	GAT	TAT	
PR145	AAT	TTT	AGT	AAT	GAT	6
TM327	AAT	TTT	AGT	AAT	GAT	10
TM335	AAT	TTT	AGT	AAT	GAT	10
TM336	AAT	TTT	AGT	AAT	GAT	10/9
TM338	AAT	TTT	AGT	AAT	GAT	10
TM342	AAT	TTT	AGT	AAT	GAT	10
TM343	AAT	TTT	AGT	AAT	GAT	10/6
TM345	AAT	TTT	AGT	AAT	GAT	10
TM346	AAT	TTT	AGT	AAT	GAT	6/7
TM347	AAT	TTT	AGT	AAT	GAT	10
TM352	AAT	TTT	AGT	AAT	GAT	10
GA3/GH2	AAT	TAT	AGT	AAT	GAT	9

The complete sequence of *pfmdr1* from four isolates (TM352, TM346, TM335 and GA3) was determined. The difference at amino acid #184 and the variability in the polyasparaginated linker region were the only differences seen from the previously published sequence [6]. The double numbers given in the Poly N column indicate variable numbers of repeats as seen on the sequencing autoradiograph and are consistent with the non-clonality of the isolates. The sequence of the intragenic 'alleles' is given for each isolate. [a]Amino acid numbers and nucleotide numbers of *pfmdr1* are as determined in reference 6. [b]Poly Ns are the number of predicted asparagines in the second polyasparaginated region of the linker domain as described in the text. [c]Sensitive is the wild-type genotype; R:SE Asia and R:S Amer are resistant Southeast Asian and South American genotypes as described in ref. 8.

TABLE III

***pfmdr1* gene copy number and mRNA expression analysis**

Isolate	*pfmdr1*	
	Copy No.	mRNA Level
W2	1	1
W2mef	3	2.4
TM335	1	1
PR145	4	3
TM327	2	1.8
TM336	2	3.3
TM338	>2	2.2
TM342	>2	ND
TM343	>2	ND
TM345	>2	ND
TM346	>2	2
TM347	>2	ND
TM352	>3	2.6
GH2/GA3	2	ND

Gene copy number and relative level of mRNA expression were determined as described in the text. The '>' symbol indicates an indeterminate gene copy number greater than the number given and probably indicates the isolates are non-clonal. ND indicates the analysis was not done on these isolates.

were full-length sequenced is shown in Fig. 1. The sequence analysis data given as 'Poly Ns' in Table II are the numbers of predicted Asn residues in the variable region. The isolates with double numbers indicate a mixed number of Asn residues as seen on the sequencing autoradiogram and are indicative of the non-clonality of the isolates.

Southern analysis To determine the gene copy number of *pfmdr1* in these isolates a quantitative Southern analysis was performed with the CSP gene as control. This analysis revealed increased gene copy number of *pfmdr1* in 10 of the 11 isolates and in GA3 (Table III). The gene copy number results represent compiled data from three separate experiments. The numbers given with the symbol '>' represent intermediate results (i.e. >2 means 2–3) which could not be easily rounded to a whole number. These intermediate results again are probably indicative of the nonclonality of the isolates. A representative autoradiogram from these studies is shown in Fig. 2. The occasional polymorphism seen in the 10–11-kb band representing the 3' region of *pfmdr1* in PR145 and GA3 have been noted in other strains as well (CMW, unpublished data).

mRNA quantitative analysis Dot blot analysis was performed in order to quantitate the level of expression of *pfmdr1* in these isolates. The results are shown in Table III. There was an increased level of *pfmdr1* mRNA expression for 6 of the 7 isolates tested when compared to the MFQ sensitive strain W2. The only isolate tested which did not show increased expression was TM335, the isolate which was sensitive to MFQ and had a single gene copy of *pfmdr1*. The level of increased expression was somewhat correlated with gene copy number, however there was no apparent correlation with the IC_{50} to MFQ in individual isolates.

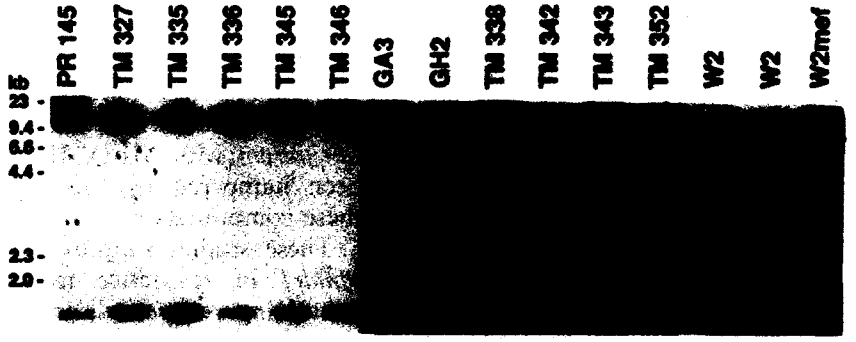

Fig. 2. Quantitative Southern Analysis. Shown is a representative autoradiograph of a Southern analysis of genomic DNA, cut with *Eco*RI, and simultaneously probed with a 3' probe for *pfmdr1* (10–11-kb bands) and a probe for the CSP gene (1-kb band) as described in the text. Isolate TM347 is not shown in this Fig.

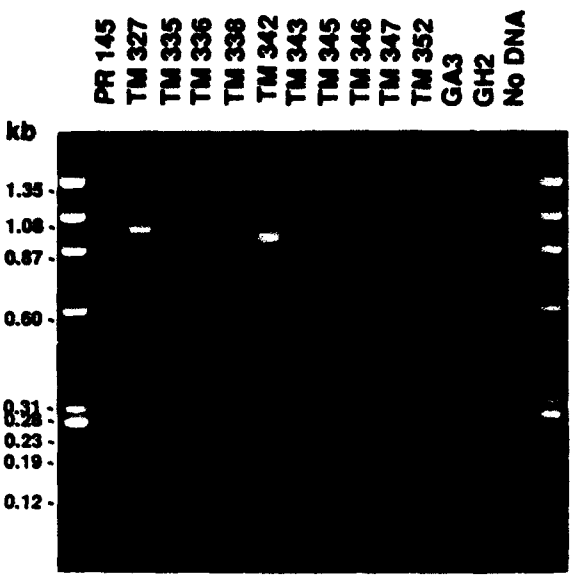

Fig. 3. Analysis of the 3' Polymorphism of *pfmdr1*. Pictured is the ethidium bromide stained gel of the 3' polymorphic region of *pfmdr1* which was amplified by PCR as described in the text.

Isolate characterization To confirm that the Thai isolates were distinct isolates, the 3' polymorphism of *pfmdr1* was amplified by PCR and a fingerprint analysis was performed.

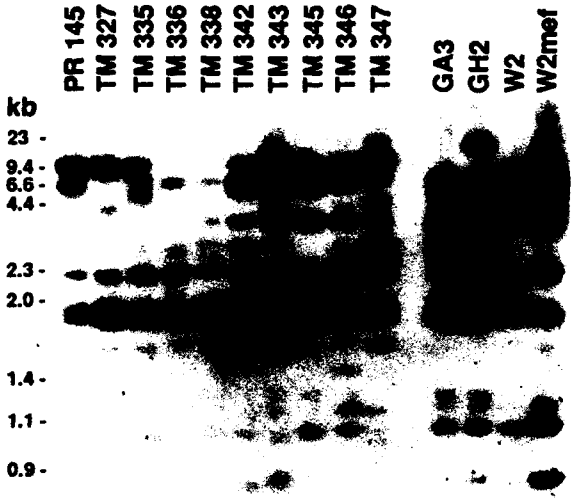

Fig. 4. Fingerprint analysis of isolates. The figure represents an autoradiograph of a Southern analysis of genomic DNA, cut with *HinfI* and probed with a chromosomally-dispersed repetitive sequence as described in the text. Isolate TM352 is not shown in this figure.

These results are shown in Figs. 3 and 4 respectively. The isolates showed considerable polymorphism by both techniques. There were no two isolates which had similar patterns by both analyses. The degree of polymorphism seen in the analysis of the 3' end of *pfmdr1* by PCR is much greater than previously reported [8].

Discussion

These isolates from Thailand show a disturbing trend in their pattern of resistance to antimalarials. That is, in addition to being resistant to CLQ, MFQ, amodiaquine, pyramethamine and QUIN, they show reduced susceptibility to HAL and QHS with no apparent exposure to these compounds. The appearance of concurrent MFQ and HAL resistance has been previously reported both in vivo [11] and in vitro [11,13,25]. More recently a pattern of concurrent MFQ, HAL and QHS reduced susceptibility has been noted [26]. This pattern is suggestive of an MDR phenotype and these concurrently acquired resistances may be clinically relevant [21]. However, the in vitro and clinical correlative data for HAL and QHS have not been developed enough to give actual predictive resistance levels.

Interestingly, resistance to MFQ, HAL, and QHS can all be modulated by penfluridol but not generally by verapamil or tricyclic compounds (DEK unpublished data). Penfluridol is a neuroleptic drug of the diphenylbutylpiperidine class which has calcium channel blocking activity [22]. There are no reported compounds which modulate both CLQ and MFQ resistance. Efflux studies in *P. falciparum* with MFQ, HAL and QHS have been hampered by the lipophilic nature of these compounds.

These studies support a potential role for *pfmdr1* in resistance to certain quinoline-containing compounds in *P. falciparum*. Our initial studies showed an increased gene copy number and increased mRNA expression of *pfmdr1* associated with the in vitro acquisition

of MFQ and HAL resistance [5]. The majority of these isolates from Thailand have an increased gene copy number (11 of 12) and increased mRNA expression (6 of 7) for *pfmdr1*. The only isolate in this sample with a single copy of *pfmdr1*, TM335, was sensitive to MFQ and HAL. Thus all of the isolates in this sample which had reduced susceptibility to MFQ and HAL had multiple copies of *pfmdr1* although the level of MFQ resistance did not directly correlate with the gene copy number. These data then suggest an association of increased gene copy number and expression of *pfmdr1* and the concurrent acquisition of MFQ and HAL decreased sensitivity. Extended studies on isolates from different geographic regions with varying resistance patterns should help clarify this association.

The sequence data from these isolates contradicts the previously reported association of intragenic alleles of *pfmdr1* and chloroquine resistance [8]. The sequence analysis of *pfmdr1* in the 11 recent isolates demonstrated only a single nucleotide change (A to T) found at nucleotide 1051 giving a predicted change of Tyr to Phe at position 184 compared to the wild-type sensitive strain [7]. This change at amino acid position 184 is in a position previously noted to be variable (8 and unpublished data CMW). Sequence analysis of the earlier isolates from the same region (GH2 and its clone GA3) revealed identical sequence to the sensitive strain. Hence all of the isolates in this study would have been predicted to be chloroquine sensitive by the criteria suggested for intragenic sequence analysis of *pfmdr1* [8] yet all were chloroquine resistant. Consequently these data contradict the only genotypic data supporting a linkage between *pfmdr1* and chloroquine resistance.

The reasons for this discrepancy are not clear. However isolates from SE Asia from 1989 analyzed by Foote et al. (ref. 8; Table I) would similarly have been predicted incorrectly had they been included in the blinded analysis. The patterns of the 3' polymorphism of *pfmdr1* from the isolates in this study are much more variable than previously indicated and could actually be used to distinguish the isolates. This may indicate the isolates tested in the original sample by Foote et al. [8] were more closely related than appreciated.

Although this data does not support a linkage between *pfmdr1* and CLQ resistance there remains strong phenotypic evidence implicating a P-glycoprotein homologue in CLQ resistance [2,3,23]. This implies that either additional or distinct genes of the ABC superfamily of genes could be involved in CLQ resistance.

The physiological role of *pfmdr1* has not been determined. Members of the MDR gene family mediating drug resistance in mammalian cells are generally overexpressed, conferring the multi-drug-resistant phenotype [24]. However it is becoming clear that variable expression and processing of certain classes and subclasses of members of the mammalian MDR gene family can lead to variable phenotypic expression. Members of the MDR gene family are part of the ABC superfamily of transport proteins [4]. Members of this superfamily have been identified in widely diverse organisms and are implicated in the transmembrane transport of a broad variety of compounds.

In summary, we have examined a number of unique recent isolates of *P. falciparum* from Thailand and found them to be resistant to multiple quinoline-containing compounds, artemisinin and pyramethamine. Decreased susceptibility to artemisinin and halofantrine have potentially developed without drug pressure. Sequence analysis of *pfmdr1* in these isolates revealed no association between polymorphisms and chloroquine resistance as had been previously reported. We describe an apparent association of increased gene copy number and overexpression of *pfmdr1* with the concurrent acquisition of MFQ and HAL reduced susceptibility. Further studies should clarify this association.

Acknowledgements

We are grateful to Lucia Gerena of WRAIR for technical assistance, Dr Tom McCutchan of NIH for the CSP clone, and Dr Tom Wellems of NIH for the pC4.H32 clone. This

work was supported by grants from the John D. and Catherine T. MacArthur Foundation, the NIH AI-27872, the UNDP/World Bank/WHO Programme for Tropical Disease Research (TDR) and U.S. Army (DAMD 17-91-C-1012) to D.F.W. C.M.W. is supported by NIAID grant K11 AI00892-04. S.K.V. received support from the NIH (T32AI-07350). D.F.W. is a Burroughs Wellcome Scholar in Molecular Parasitology.

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MOLBIO 01876

Stage-specific transcripts of the *Plasmodium falciparum pfmdr1* gene

Sarah K. Volkman, Craig M. Wilson* and Dyann F. Wirth

Department of Tropical Public Health, Harvard School of Public Health, Boston, MA, USA

(Received 29 June 1992; accepted 12 September 1992)

Genes have been identified in *Plasmodium falciparum* which belong to the ATP-binding cassette superfamily of transport systems based upon sequence and structural homology. One of these genes, the *pfmdr1* gene, is homologous to the mammalian *mdr1* gene, which encodes the P-glycoprotein product. Strong phenotypic data suggests the involvement of a P-glycoprotein-like molecule in the mediation of drug resistance in *P. falciparum*. The goal of this work was to characterize the expression of the *pfmdr1* messenger RNA: both the stage specific expression and the level of expression in mefloquine sensitive or resistant parasites. We identified two messenger RNAs homologous to this gene, one of 8.5 kb expressed in ring and trophozoite stages, and a second messenger RNA of 7.5 kb expressed only in trophozoites. Previously we had reported an increased expression of messenger RNA for *pfmdr1* in a mefloquine-resistant clone. Here we extend this and demonstrate that overexpression of the *pfmdr1* gene is consistent with the mefloquine resistance phenotype in a panel of recent isolates from Thailand.

Key words: Malaria; *Plasmodium falciparum*; Drug resistance; Multiple-drug resistant; Mefloquine

Introduction

Members of an extended ATP-binding cassette superfamily of transport systems (reviewed in references 1–4) have been identified in such evolutionarily diverse organisms as bacteria [1], yeast [5], and man [6,7]. The functions of these gene products involve metabolite transport in bacteria [1], pigment transport in *Drosophila* [8], and pheromone transport in yeast [5]. In addition, members of this supergene family have been implicated in anion transport in cystic fibrosis [7], in modulation of antigen presentation by the class I major histocompatibility complex [9], and in the mediation of drug resistance in mammalian cells (for review see ref. 6).

The genes involved in mediating drug resistance in mammalian cells are part of the multiple drug resistance or MDR gene family. The MDR phenotype is typically characterized by amplification of a large segment of DNA or overexpression of messenger RNA (mRNA) encoding the P-glycoprotein product [6]. In addition, members of this gene family have been shown to be overexpressed in drug-resistant cell lines and can confer multiple drug resistance when transfected and over-expressed in otherwise drug-sensitive cells [10].

Genes (*pfmdr1* and *pfmdr2*) have been identified in the human malaria *Plasmodium falciparum* which are members of this superfamily based upon sequence and structural homology [11,12]. Strong phenotypic data suggests the involvement of a P-glycoprotein-like molecule in the mediation of drug resistance in *P. falciparum*, since drug resistance has been shown to involve an efflux mechanism [13] and calcium channel blockers

Correspondence address: Dyann F. Wirth, Dept. of Tropical Public Health, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115, USA.

*Present address: Division of Geographic Medicine, University of Alabama at Birmingham, Birmingham, AL, USA.

Abbreviations: HAL, halofantrine; MFQ, mefloquine; CLQ, chloroquine; MDR, multiple-drug resistant.

such as verapamil or penfluridol can reverse this efflux mechanism [13–16]. The question of what role these genes may play in drug resistance in malaria is currently under investigation.

Drug resistance in *P. falciparum* may be characterized by three distinct patterns, based upon either class of drug (sulfadioxide or aminoquinoline) or cross-resistance pattern (chloroquine (CLQ) versus mefloquine (MFQ)). These classes of drug resistance can be identified by the antimalarials (1) pyrimethamine, (2) CLQ, and (3) MFQ. The mechanism of pyrimethamine resistance is known to involve a point mutation in the dihydrofolate reductase-thymidylate synthetase gene of the malaria parasite [17,18]. CLQ resistance is characterized by an efflux mechanism such that a drug resistant organism can expel CLQ more rapidly than a drug sensitive one [13]. The mechanism of CLQ as an antimalarial involves the ability of CLQ to interfere with a heme detoxification enzyme that is required for parasite survival in the erythrocyte [19]. Research investigating the possible role of *pfmdr1* in CLQ resistance over the last few years has produced some apparently conflicting results. Increased gene copy number does not correlate with CLQ resistance [11]; and, although polymorphisms have been detected in the *pfmdr1* gene [20] which were thought to associate with CLQ resistance, work in our laboratory [30] has shown that these polymorphisms are not found in CLQ-resistant (CLQR) parasites recently isolated from Thailand. Most importantly, a genetic cross between a CLQ-sensitive (CLQS) and a CLQR strain has demonstrated that the *pfmdr1* gene can be dissociated from the resistance and reversal phenotype in the progeny [21,22]. This result suggests that another transporter, perhaps another member of the MDR family, is involved in this resistance. Additional molecular characterization is required to resolve this point.

MFQ resistance is distinct from CLQ resistance, having characteristics that may be more similar to the mammalian MDR phenotype. These features include: the pattern of cross resistance, the reversal modulator involved, and the rate at which resistance occurs. MFQ resistance is often associated with halofantrine (HAL) resistance. MFQ-resistant (MFQR) isolates from geographic regions where HAL and artemisinin are unavailable show this cross-resistance pattern [23]. Penfluridol has been shown to reverse MFQ resistance and recent evidence has demonstrated that it also reverses HAL resistance, indicating a common resistance mechanism (D. Kyle, personal communication). Furthermore, our recent work has demonstrated an association of increased gene copy number with MFQ resistance both in laboratory isolates and in recent field isolates [30].

One of the goals of this work is to determine if *pfmdr1* mRNA expression correlates with MFQ resistance. Overexpression of *pfmdr1* mRNA has been associated drug resistance [11,12], but this analysis has been limited to a comparison between two pairs of drug sensitive and resistant parasites. This work extends this study and analyzes the levels of *pfmdr1* mRNA in both a pair of laboratory clones differing by MFQ resistance and a panel of field isolates from mefloquine treatment failures in Thailand in terms of their MFQR phenotype. Previously, we reported a single transcript for this gene of between 7 and 8 kb that appeared to be overexpressed in W2mef as compared to W2 [12]. In the work reported here a second transcript for the *pfmdr1* gene has been identified. These two transcripts are stage specifically expressed, and are larger than predicted for the gene sequence [11] or the protein product [24]. The findings in this paper are then discussed in the context of how RNA expression may be related to MFQ resistance in *P. falciparum*.

Materials and Methods

Parasite cultures. Parasites were cultivated in vitro by a modification of the method of Trager and Jensen [25]. Isolates were maintained in a 5% suspension of type A+ erythrocytes in RPMI 1640 supplemented

with 32 mM $NaHCO_3$/12 mM Tes/37 mM hypoxanthine/2 mM glutamine/10 mM glucose/10% human plasma in an atmosphere of 1% O_2/5% CO_2/balance nitrogen. Synchronization of parasite cultures was performed with either 5% sorbitol [26] or incubation with aphidicolin (1.5 $\mu g\ ml^{-1}$ final), which inhibits DNA polymerase, for 20 h [27]. This drug was subsequently removed allowing continued maturation of the trophozoites to schizonts followed by reinvasion of the merozoites into erythrocytes in a synchronized manner.

Hypoxanthine-uptake assay. Parasite cultures were placed in 96-well assay dishes at 1% parasitemia and 2% suspension of erythrocytes in supplemented RPMI containing dilutions of drug concentrations in triplicate sets. Parasites were incubated for 18–24 h as described above before 1 μCi [^{3}H]hypoxanthine (Amersham) was added to each well. After an additional 18-h incubation, assay plates were harvested in a Scantron harvester onto glass fiber filters for determination of the number of counts min^{-1}.

RNA extraction. Cultivated parasites were washed in RPMI and resuspended in lysis buffer (50 mM Hepes/2 mM EDTA/100 mM NaCl). Sarkosyl (10%) was added to a final concentration of 1% followed by vigorous mixing. The aqueous phase was repeatedly extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) until no interface material remained. The nucleic acid was precipitated with ethanol after the addition of sodium acetate (pH 5.2) to 0.3 M. RNA from stage-specific parasites was isolated after synchronization.

Poly(A)$^+$ isolation. Total RNA isolated as described above was subjected to oligo(dT) chromatography as described by the manufacturer (Pharmacia). In brief, 500 μg of total mixed stage RNA was heat denatured, resuspended in sample buffer (10 mM Tris-HCl, pH 7.4/1 mM EDTA/500 mM NaCl), applied to an equilibrated oligo-(dT)-cellulose spun column. The column was then washed with high-salt buffer (10 mM Tris-HCl, pH 7.4/1 mM EDTA/500 mM NaCl) followed by low-salt buffer (10 mM Tris-HCl, pH 7.4/1 mM EDTA/100 mM NaCl) at 350 × *g*. Eluate was collected from four applications of elution buffer (10 mM Tris-HCl, pH 7.4/1 mM EDTA) and concentrated by precipitation in ethanol with the addition of NaCl to 0.3 M and glycogen to 0.1 mg/ml.

Enzymatic treatment of RNA. RNA samples were subjected to enzymatic treatment with RNase-free DNase (RQ1; Pharmacia) or DNase-free RNase for 10 min at 37°C before the addition of 5 mM EDTA, pH 8.0.

Northern analysis. Equal amounts of total nucleic acid (10 μg) or poly(A)$^+$ RNA (1 μg) were resolved on a 1.3% agarose gel in 0.66 M formaldehyde. The RNA was transferred to nitrocellulose membranes and hybridization was performed either with the *pfmdr1* probe (corresponding to bases 4400–4742; ref. 11), or a ribosomal RNA probe. Probes were radiolabeled using the random oligonucleotide priming technique as described [28]. Hybridization was performed in 1% SDS/1 M NaCl/10% dextran sulfate/50% formamide at 42°C overnight. Autoradiograms were made of the RNA blots.

Quantitation. RNA was quantitated by both densitometry of Northern blots and by directly counting regions of hybridized dot blots. Laser densitometry of Northern blots was performed using the Bio Image system (Millipore). Quantitation by dot blot analysis was done by counting the regions of nitrocellulose directly in a β-scintillation counter. Total nucleic acid (20 μg) was resuspended in denaturing buffer (50% formamide/6.6% formaldehyde/1 × SSC (SSC = 150 mM NaCl/15 mM Na_3 citrate · $2H_2O$, pH 7)) and heated to 65°C for 15 min. Two vols. of 20 × SSC were added and equal amounts of sample were loaded onto replicate nitrocellulose filters that had been repeatedly washed with 10 × SSC. The samples were allowed to remain 30 min before vacuum was applied. Replicate filters were then hybridized as described above

TABLE I

Quantitation of W2 and W2mef *pfmdr1* RNA expression

Strain	CLQ (ng/ml)	MFQ (ng/ml)	Total	Ring	Troph
HB3	S (6–8)	S (4)	1 ± 0.5	1 ± 0.5	1 ± 0.6
W2	R (70–90)	S (2)	1.2 ± 0.3	0.7 ± 0.1	1.6 ± 0.5
W2mef	R (30–60)	R (15–20)	2.9 ± 0.5	2 ± 0.4	3.4 ± 1.7

Multiple samples of HB3, W2 and W2mef total and stage specific RNA were analyzed by slot hybridization with either the *pfmdr1* or the ribosomal probe. The values shown represent the ratio of *pfmdr1* to ribosomal signal which were normalized to the HB3 values (± standard deviation). Median inhibitory concentrations for CLQ and MFQ are included.

with either the *pfmdr1* or ribosomal RNA probe. Counts corresponding to the *pfmdr1* signal were adjusted for loading using the ratio of these values with the values for the ribosomal RNA probe counts and normalizing these ratios to those of the sensitive indicator strain. Standard deviations were determined for the values shown in Table I, since 3–24 samples were analyzed by both Northern and dot blot methods. Values shown in Table II are derived from an average of 2 separate Northern blots and 2 dot blots; therefore, statistics were not determined on these averages. A representative Northern (see Fig. 6) is shown, and is consistent with these averages.

TABLE II

Quantitation of MFQS or MFQR isolates from Thailand

Strain	MFQ (ng/ml)	Gene copy number	Total RNA
W2	S (2)	1	1
W2mef	R (15–20)	3	2.4
TM335	S (6)	1	1
PR145	R (10)	4	3.4
TM327	R (16)	2	2.1
TM336	R (15)	2	2.7
TM338	R (9)	>2	2.9
TM346	R (20)	>2	2
TM352	R (22)	>3	1.7

Total RNA from some recent isolates from Thailand along with the W2 strain was analyzed by slot hybridization. Median inhibitory concentrations for MFQ and *pfmdr1* gene copy number [12,30] are shown. Values are expressed as the ratio of *pfmdr1* to ribosomal signal, normalized to W2.

Results

Previously we had identified a single transcript of between 7 and 8 kb for the *pfmdr1* gene [12], while Foote et al. [11] identified 4 transcripts of 6.5 kb, 5.4 kb, 4.2 kb and 1.9 kb. Both groups reported overexpression of *pfmdr1* RNA in a single pair of drug-sensitive or resistant strains. To resolve the discrepancy regarding the number and size of *pfmdr1* transcripts, to further characterize the expression of the mRNA for the *pfmdr1* gene, and to

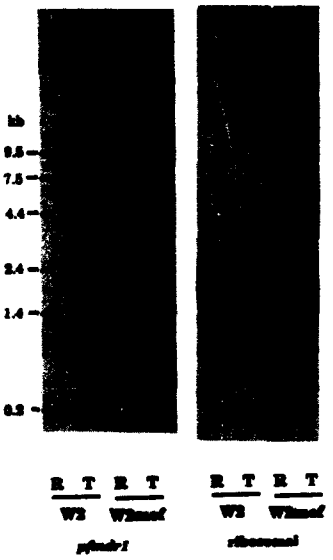

Fig. 1. Hybridization analysis of stage-specific W2 or W2mef RNA. RNA from sorbitol-synchronized cultures was resolved on a formaldehyde agarose gel. The nucleic acid was then transferred to nitrocellulose and hybridized with first the *pfmdr1* probe and then with the ribosomal probe. RNA samples from both ring (R) and trophozoite (T) stage cultures of W2 and W2mef are shown as autoradiograms after hybridization.

determine if overexpression of *pfmdr1* RNA was associated with MFQ resistance, we performed Northern analysis of RNA samples.

We began by examining steady state levels of *pfmdr1* RNA from both a MFQ sensitive (MFQS) and a MFQR strain of *P. falciparum* to determine the number and size of the transcripts, and to determine if there was any correlation with MFQ resistance. A clone of W2, a CLQR *P. falciparum* isolate from Indochina, was placed under stepwise MFQ pressure in vitro to derive W2mef, its clonal daughter strain which is CLQR and MFQR [29]. A probe for the *pfmdr1* gene was found to hybridize to 2 transcripts in total RNA samples from mixed stage erythrocytic cultures. To determine whether these two transcripts were stage specifically expressed, we performed Northern analysis of sorbitol synchronized parasite cultures (Fig. 1). In samples derived from the early ring erythrocytic stage, a single transcript of 8.5 kb was present. In samples derived from the later trophozoite erythrocytic stage, an additional 7.5-kb transcript was present. This pattern of a single transcript in ring stages and two transcripts in trophozoite stages was present in both W2 and W2mef. A ribosomal probe was used to determine relative levels of sample loading. There was a higher level of expression of both transcripts in W2mef as compared to W2 for both stages.

To confirm the stage specific expression observed, we wanted to examine the expression of these transcripts in a time-dependent fashion throughout the erythrocytic cycle. Parasite cultures were tightly synchronized by incubation with aphidicolin. Fig. 2 shows a Northern blot of representative time points from such a synchronization. There was a single 8.5-kb transcript in ring stages and two transcripts of 7.5 and 8.5 kb in trophozoite stages. Interestingly, in the schizont stage, the level of both *pfmdr1* transcripts was significantly reduced, when loading levels were adjusted using the ribosomal RNA probe, as compared to other stages of the cycle.

To establish that these RNA molecules were polyadenylated, we subjected samples to

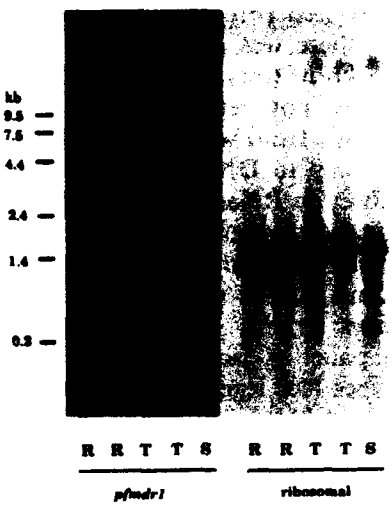

Fig. 2. Time course analysis of *pfmdr1* RNA expression in W2mef. RNA samples from aphidicolin-synchronized W2mef parasite cultures were taken at various times from the ring (R), trophozoite (T) or schizont (S) stages and analyzed as in Fig. 1. Autoradiograms of a Northern blot probed with first the *pfmdr1* then the rDNA probe is shown.

oligo(dT) chromatography. Both of these transcripts are retained by oligo(dT) in chromatography experiments, since hybridization was present to both transcripts when the oligo(dT) bound fraction was subjected to Northern analysis, and was absent in oligo(dT) unbound fractions under similar conditions, indicating that these transcripts were

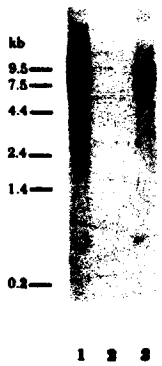

Fig. 3. Oligo(dT) analysis of the two *pfmdr1* transcripts. Total mixed stage W2mef RNA was run as either unfractionated (lane 1), oligo(dT)-unbound fraction (lane 2), or oligo(dT)-bound fraction (lane 3) and analyzed as above using the *pfmdr1* probe.

polyadenylated (Fig. 3). In this fractionation analysis, there was a hybridizing band larger than 9.5 kb in total nucleic acid preparations which was not retained by the oligo(dT) matrix. This band was shown to be DNA by its sensitivity to DNase (Fig. 4, lane 2), and insensitivity to RNase (Fig. 4, lane 3).

To analyze whether the expression of the two *pfmdr1* transcripts observed for the W2 and W2mef strains was consistent in other *P. falciparum* strains, total mixed stage RNA from a panel of isolates with different drug phenotypes (see legend, Fig. 5) was subjected to Northern blot analysis and hybridized with the *pfmdr1* probe. The autoradiograms from this analysis (Fig. 5) indicate that two transcripts were observed in multiple strains with different drug sensitivity and resistance profiles. This implies that the presence of 2 transcripts is a general feature of this gene expression.

To determine the level of expression of these stage specific transcripts in drug resistant as compared to drug-sensitive strains, quantitation of RNA levels was done by dot blot analysis. Previously we found that the observed *pfmdr1* transcript was apparently overexpressed in W2mef as compared to W2 [12]. To confirm these observations and to determine if there was a stage-dependent expression of the two transcripts identified in this work, extensive analysis was performed on multiple samples from each strain and stage (see Fig. 1 and Table I). RNA samples from total mixed stage erythrocytic cultures along with samples from stage synchronized cultures were hybridized with either the *pfmdr1* or the ribosomal probe and the hybridization results were quantified. Table I shows a summary of these results using the drug sensitive HB3 strain as a comparison. Values for the ratio of hybridization signal of the *pfmdr1* probe relative to the rDNA probe are greater for W2mef as compared to W2 when normalized to HB3.

This analysis examined levels of *pfmdr1* mRNA expression in a pair of clonal laboratory strains that differ by resistance to MFQ as a result of MFQ pressure in vitro. We wanted to determine if this was consistent with strains of parasites that were MFQR as a result of natural drug pressure. We therefore extended this quantitative analysis to a panel of recent isolates from Thailand [30]. Six of the isolates are resistant to MFQ by in vitro testing and one isolate from the same geographic region, TM335, is sensitive to MFQ. A representative Northern blot with RNA from some of these isolates is presented in Fig. 6, showing *pfmdr1* signal along with ribosomal signal for loading

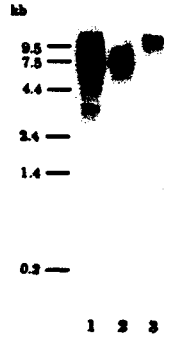

Fig. 4. RNase and DNase analysis of the two *pfmdr1* transcripts. Total W2mef RNA was incubated with no enzyme (lane 1), RNase-free DNase (lane 2) or DNase-free RNase (lane 3) and the resulting nucleic acid was resolved on formaldehyde agarose gels, transferred, and probed with the *pfmdr1* molecule.

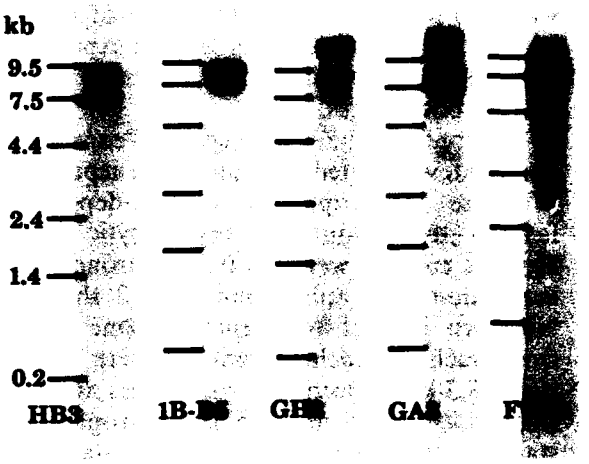

Fig. 5. Two *pfmdr1* transcripts are present in multiple strains. RNA samples from different isolates (HB3 (CLQS; MFQS), FCR3 (CLQR; MFQS), GH2 (CLQR; MFQR), GA3 (CLQR; MFQR), and 1B-B5 (CLQR; MFQS)) were resolved on denaturing agarose gels, transferred and hybridized with the *pfmdr1* probe. Each Northern is shown with its own markers as they were run on separate gels.

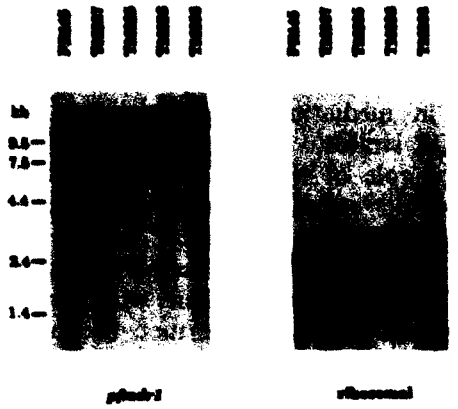

Fig. 6. Northern analysis of MFQS or MFQR isolates from Thailand. RNA from unsynchronized cultures was resolved on a formaldehyde agarose gel. The nucleic acid was analyzed as described, and are shown as autoradiograms after hybridization with either the *pfmdr1* probe or the ribosomal probe.

levels. A combination of Northern and dot blot analysis on multiple RNA samples (see Materials and Methods) is summarized in Table II. All of these recent MFQR isolates from Thailand had increased expression of the *pfmdr1* gene when compared to the recent MFQS isolate TM335 or the W2 strain. We find that an increased level of *pfmdr1* mRNA is consistent with a MFQR phenotype in this panel of isolates.

Discussion

We have identified two polyadenylated transcripts for the *pfmdr1* gene which are stage specifically expressed throughout the erythrocytic cycle of the parasite. Analysis of this expression demonstrates that an 8.5-kb transcript is present in all stages of the erythrocytic cycle and that an additional 7.5-kb transcript is present in the trophozoite and schizont stages of this cycle. In addition, these transcripts are overexpressed in MFQR as compared to MFQS strains, both in a laboratory derived resistant clone as well as in some recent field isolates from Thailand. Thus, overexpression of *pfmdr1* mRNA is consistent with MFQ resistance in the strains analyzed.

Our previous work [12] had shown that there was a single transcript for the *pfmdr1* gene that was approximately 8 kb in size. With well resolved Northern analysis we are able to observe two transcripts that migrate at 8.5 kb and 7.5 kb, based upon Northern analysis using the *pfmdr1* probe. These two transcripts are present in RNA from every strain examined, regardless of drug sensitivity or resistance phenotype. These data are inconsistent with the Northern results presented by Foote et al. [11], which suggest that there are 4 transcripts encoded by the *pfmdr1* gene. Using a probe to the same region of the gene, we find cross hybridization to products which migrate with the ribosomal subunits. These cross-hybridizing products are not retained by oligo(dT) and are DNase-resistant and RNase-sensitive, suggesting that they are not polyadenylated but that they are RNA in nature (SKV, unpublished). In addition, expression levels for the *pfmdr1* transcripts are greater in the trophozoite stage relative to the ring stage for both W2 and W2mef; however, in schizont stage levels are markedly reduced relative to the trophozoite stage. This is different from the observations of Foote et al. [11], where expression in schizont stage seemed increased as compared to trophozoite stage, but based on our analysis, we believe that the Foote et al. data [11] represent cross-hybridization to ribosomal RNA.

The structural nature of these transcripts and the mechanism by which they are derived has yet to be established. The two transcripts differ in size from one another and from the size either the open reading frame [11] or the protein product identified [24] would predict. The structural differences suggest that either the two mRNAs are processed to give one protein product, or that the two transcripts encode distinct protein products. Perhaps these structural differences may be functionally important, for example, in regulating expression of these transcripts or the resulting proteins, mediating the interaction of these products with other gene products that may be involved in drug resistance, or targeting these products to their appropriate sites of action.

We have shown that these transcripts are expressed in drug sensitive as well as drug resistant strains; therefore, it is unlikely that their expression determines drug resistance. In the mammalian system it is known that multiple transcripts may be encoded by a single gene, but that the resulting products code for proteins that specify different drug resistance phenotypes [6]. Perhaps RNA processing is important in determining the drug resistance phenotype, either regulated at the RNA level, or specified by the resulting proteins. Overexpression of the mRNA for the *pfmdr1* gene, which corresponds to an increase in gene copy number [30], is consistent with a MFQR phenotype in the strains examined. This suggests that gene amplification and overexpression of RNA, like in the mammalian MDR system, may be an important mechanism of MFQ resistance in *P. falciparum*.

These two transcripts might code for distinct protein products, but the protein data for *pfmdr1* suggests a single product. This product, Pgh1, has been determined as a 160-kDa protein which is present throughout the erythrocytic cycle [24]. This product is localized to the parasite food vacuole only during the trophozoite stage of the erythrocytic cycle [24]. The appearance of the 7.5-kb transcript in the trophozoites stage is consistent with this localization of Pgh1 to the food vacuole.

Overexpression of *pfmdr1* mRNA is consistent with MFQ resistance in both the laboratory clones (W2 versus W2mef) and in a panel of recent field isolates from Thailand. This suggests that one mechanism of MFQ resistance may involve overexpression of *pfmdr1* mRNA. Increased *pfmdr1* RNA levels in the MFQR W2mef strain as compared to the MFQS W2 parent strain demonstrated in this work is consistent with the finding that W2mef has multiple copies of the *pfmdr1* gene whereas W2 has only a single copy [12]. Furthermore, when we extend this analysis to recent field isolates from Thailand, we find that increased *pfmdr1* gene copy number is associated with MFQ resistance [30] and overexpression of the *pfmdr1* RNA accompanies this increase in gene copy number in these isolates. The results therefore support the notion that overexpression of the *pfmdr1* gene product may play a role in mediating drug resistance in some MFQR strains.

The role of the *pfmdr1* gene and its two transcripts remain to be determined, but by analogy with the function of members of this gene family in other systems, we hypothesize that the encoded protein will function in membrane transport. Further, because of the association of increased gene copy number [12,30] and increased expression of this mRNA with MFQ resistance, we suggest that this gene may be involved in MFQ resistance. Proof of this awaits functional analysis.

Acknowledgements

This work was supported by grants from the John D. and Catherine T. MacArthur Foundation, the NIH (AI-27872), the UNDP/World Bank/WHO Programme for Tropical Disease Research (TDR) and U.S. Army (DAMD 17-91-C-1012) to D.F.W. S.K.V. received support from the NIH (T32AI-07350). C.M.W. is supported by NIAID Grant No. K11 AI00892-04. D.F.W. is a Burroughs Wellcome Scholar in Molecular Parasitology.

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Molecular and Biochemical Parasitology, 60 (1993) 195–208

MOLBIO 02802

Cloning and functional analysis of an extrachromosomally amplified multidrug resistance-like gene in *Leishmania enriettii*

Larry M.C. Chow[a], Alexander K.C. Wong[a], Buddy Ullman[b] and Dyann F. Wirth[a]

[a]*Department of Tropical Public Health, Harvard School of Public Health, Boston, MA, USA; and* [b]*Department of Biochemistry and Molecular Biology, Oregon Health Science University, Portland, OR, USA*

(Received 4 January 1993; accepted 9 April 1993)

The goal of this work was to investigate the mechanism of drug resistance in *Leishmania enriettii* as a model system for drug resistance both in human leishmaniasis and on other parasitic diseases. Parasites were selected in increasing concentrations of vinblastine, an inhibitor of microtubule assembly, and resistant clones were isolated which grew in concentrations 5–30 times the IC_{50} (30 $\mu g\ ml^{-1}$) of parental cells. The vinblastine-resistant parasites were also resistant to puromycin, an unrelated drug which inhibits protein synthesis. This cross-resistance to unrelated drugs had previously been observed in mammalian cells and recently in *L. donovani*. The proposed mechanism for this cross-resistance is drug efflux mediated by increased expression of a P-glycoprotein molecule encoded by a multidrug resistance (*mdr*) gene. Here we report the identification, cloning and sequencing of an *mdr*-like gene from *L. enriettii*, *lemdr1*, and demonstrate that this gene is amplified on an extrachromosomal circle of 35–40 kb in vinblastine-resistant *L. enriettii*. The longest open reading frame in the cloned gene is 1280 amino acids with a predicted protein of 140 kDa. The predicted protein has a structure similar to that for all other reported P-glycoproteins namely 12 transmembrane domains and 2 ATP binding sites, arranged in 2 similar half-molecules. Comparison of the primary amino acid sequence with other known *mdr* gene products demonstrates a significant homology with 37% amino acid identity with human *mdr1* and 83% identity with the *L. donovani ldmdr1* gene. The *lemdr1* gene was cloned in the expression vector pALTNEO and transfected into wild-type *L. enriettii* and the resulting transfected cells were resistant to vinblastine but at lower levels than in the selected mutant cells.

Key words: *Leishmania*; Drug resistance; Amplification; Transfection

Introduction

Drug resistance is emerging as a major problem in many parasitic diseases. It may pose a significant problem for treatment and control of these diseases and their resulting morbidity and mortality. Recent evidence has suggested that drug efflux mediated by P-glycoprotein-like membrane transporters, encoded by multidrug resistance (*mdr*) gene, may play a role in drug resistance mechanisms in several protozoan parasites. The goal of this work is to investigate the role of such *mdr*-like genes recently identified in several protozoan parasites in drug resistance. This work will focus on *Leishmania enriettii* as a model system and will serve as a basis for a more generalized application to other protozoan parasites.

The phenomenon of multidrug resistance was first described in mammalian neoplastic cells derived either in vitro through step-wise selection in various anti-neoplastic drugs or in tumors which became refractile to chemother-

Correspondence address: Dyann F. Wirth, Department of Tropical Public Health, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115, USA. Tel: (617) 432-1563; Fax: (617) 738-4914.

Note: Nucleotide sequence data reported in this paper have been submitted to the GenBank™ data base with the accession number L08091.

Abbreviations: mdr, multidrug resistance; TAFE, transverse-alternating field electrophoresis.

apy [1–4]. Neoplastic cells subjected to treatment with a single drug became resistant to multiple, unrelated drugs through a common mechanism of drug efflux mediated by overexpression of a membrane transport protein, the P-glycoprotein. The primary role of this P-glycoprotein mediated mechanism of drug resistance has been demonstrated by transfection of drug sensitive cells with the cDNA of the *mdr* gene [2]. *mdr* homologues have been found in other organisms, including mouse [5–8], rat [9], hamster [10], yeast [11,12], *Drosophila* [13] and some protozoans [14–18]. They all shared the same putative structures, namely an internal duplication with each half having 6 transmembrane-domains (TM) and one highly conserved nucleotide binding motif. It is postulated that the P-glycoproteins originated from the duplication of the bacterial membrane transporter genes [3,19,20].

Although it is well established that the P-glycoprotein is responsible for the multidrug resistance in mammalian cells, the role of this gene in normal cells has yet to be determined. Evidence from analysis of drug efflux and the similarity of the P-glycoprotein structure with bacterial transport proteins has led to the hypothesis that the P-glycoprotein is directly involved in the transport of drugs from the cell. In support of this hypothesis, recent work has demonstrated that the mouse *mdr* gene can functionally complement the *ste6* in yeast for the transport of pheromone [12]. Second, reconstitution experiment showed that the P-glycoprotein has a drug-inducible ATPase activity [21]. Recently, the P-glycoprotein was shown to have a chloride-selective channel activity which was volume-regulated and ATP-dependent [22]. The chloride ion channel activity can be dissociated from the drug-binding activity of the P-glycoprotein [23].

mdr-like genes have been studied recently in some parasitic protozoan, including *Plasmodium falciparum* [14,15,24–26], *Entamoeba histolytica* [16,27], *Leishmania tarentolae* [17] and *Leishmania major* [28]. In *L. tarentolae* and *L. major*, an *mdr*-like gene (*ltpgpA* and *lmpgpA* respectively) was found to be amplified on an extra-chromosomal circle (H-circle) when the *Leishmania* cells were selected with various agents like methotrexate or arsenite [29–32]. In *L. major*, transfection experiments showed that the *lmpgpA* is responsible for the resistance to arsenite and some antimonial drugs but not to the hydrophobic MDR drugs like vinblastine or puromycin [28] to which mammalian MDR cells are resistant.

The goal of this work is to develop a system for the functional analysis of P-glycoprotein-like molecules in protozoan parasites in order to understand the role they may play in drug resistance mechanisms in these parasites. In previous work, we identified and partially characterized several *L. enriettii* sequences related to mammalian *mdr* genes [18]. We demonstrated a role for one of these genes in vinblastine resistance in the closely related *Leishmania donovani*. Here we report the identification, cloning and sequencing of an *mdr*-like gene in *L. enriettii* and have demonstrated that this gene is amplified on an extrachromosomal circle in parasites selected for resistance to vinblastine. In addition, we tested the function of this gene by transfection analysis. In parallel experiments, a similar gene has been identified and characterized in *L. donovani* [54].

Materials and Methods

Cell culture. *L. enriettii* promastigotes were cultivated in Schneider's *Drosophila* medium supplemented with 15% heat-inactivated fetal bovine serum (Gibco/BRL, MD), 4 mM L-glutamine, 0.04% gentamicin sulfate (Sigma, MO) at 28°C. Vinblastine-resistant cell lines were selected by exposing wild-type *L. enriettii* to a stepwise increase in drug concentration from 80 to 900 $\mu g\ ml^{-1}$ over a period of about 9 months. LeV80, LeV120, LeV160, LeV400 and LeV900 are vinblastine-resistant cells growing at a concentration of 80, 120, 160, 400 and 900 $\mu g\ ml^{-1}$ of vinblastine respectively. Vinblastine sulfate was obtained from Sigma.

Library screening. The PCR product

LEMDR06 was used as a probe to screen a *L. enriettii* genomic library which was prepared in the phage vector λ-Fix (Stratagene, CA). The LEMDR06 PCR product was derived as previously described [18]. Plaques were purified through 2 rounds of plating and 2 positive clones were obtained. They were designated as λ3 and λ99. All phage manipulations and DNA preparations were done according to the protocols described by Maniatis et al. [35].

Southern hybridization. Genomic DNA was digested with various enzymes under conditions recommended by the supplier (New England BioLabs, MA). The digested DNA was fractionated on 0.8% agarose gels and transferred to GeneScreen Plus nylon membranes (DuPont, PA) by the method of Southern [35,36]. Probes from various regions of the λ clones were labeled with [α-^{32}P] dATP as previously described [33,37]. The signals were visualized by autoradiography at −70°C.

Pulsed field gel electrophoresis. Chromosomal DNA was prepared by first embedding log phase *L. enriettii* cells in 0.5% lowmelt agarose followed by digestion with proteinase K (2 mg ml^{-1}). The blocks containing protein-free DNA was fractionated on 1% FastLane agarose (FMC, ME) gel by Transverse Alternating Field Electrophoresis (TAFE). *Saccharomyces cerevisiae* chromosomes or λ ladder were used as size markers (New England BioLabs, MA). The condition used for the separation of the linear DNA was 150 mA, 1-min pulse for 24 h at 12°C. For circular DNA, the pulse time was reduced to 4 s for 12 h. In the γ-irradiation experiment, the agarose blocks derived from LeV160 cells were suspended in 1 ml of 1 mM Tris-HCl, pH 7.6/ 50 mM EDTA. The blocks were then exposed to ^{60}Co (ICN model GR-9, CA) for various dosages up to 200 krad before analysis by TAFE.

DNA sequence and computer analysis. Double-stranded DNA sequencing was carried out with the Sequenase kit (United States Biochemical Corp., OH) based on the dideoxynucleotide chain termination method of Sanger [38]. The 2.6-kb *Pst*I fragment, 5.5-kb *Hin*cII fragment from λ3 and the 8-kb *Not*I fragment from λ99 were subcloned into pBluescript (Stratagene, CA). Sets of unidirectionally deleted clones were generated using the mung bean/ExoIII digestions (Stratagene, CA). To facilitate sequencing, primers other than the M13 universal primers were also used. Analysis of the *lemdr1* sequence was performed using software programs from PC/Gene software package (IntelliGenetics, CA) and the Genetics Computer Group software (GCG, Univ. of Wisconsin; ref. 39). Database searching was performed at the National Center of Biotechnology Information (NCBI) using the BLAST network services [40]. The hydropathy plot was generated with algorithm devised by Kyte and Doolittle [41] using a window size of 9 amino acids.

Plasmid construction. The 7-kb *Xho*I fragment containing the *lemdr1* sequence was obtained as follows: A 5.5-kb *Hin*cII fragment from λ3 was cloned into pBluescript. This plasmid (pL3H2) was digested with *Not*I and the resulting 6.2-kb fragment containing the 5′ end of the *lemdr1* gene and the pBluescript plasmid was gel purified. The 8-kb *Not*I fragment from λ99 was ligated into this vector resulting in the pCn7 plasmid containing the entire *lemdr1* coding sequence and flanking regions of DNA. The *Not*I junction region was confirmed by DNA sequence analysis. To obtain pNEOU2, the 7-kb *Xho*I fragment from pCn7 was gel purified and ligated into pALTNEO [33] which had been digested with *Xho*I under conditions of partial digestion. The structure was confirmed by restriction analysis and DNA sequence analysis.

Transfection. Late log *Leishmania* cells were harvested and washed twice in cold phosphate buffer saline. For a single transfection, 10^8 cells were resuspended in 0.5 ml of ice cold high ionic strength electroporation buffer (21 mM Hepes/ 137 mM NaCl/ 5 mM KCl/ 0.7 mM Na_2HP0_4/ 6 mM glucose, pH 7.05). Electroporations were done with 50–100 μg of

supercoiled pALTNEO or pNEOU2 DNA using the setting of 3 kV cm^{-1} and 25 μF capacitance [33]. After overnight growth, cells were selected in Schneider's medium supplemented with geneticin (50 $\mu g\ ml^{-1}$).

Drug sensitivity assay. 10^5 to 10^6 cells growing at log phase were put into the medium containing various concentrations of vinblastine or puromycin. A_{600} was measured after 3–4 days of growth. Drug sensitivity was expressed as the percentage of growth compared to the same cell line growing in the absence of drugs.

Results

Vinblastine-resistant L. enriettii are also resistant to puromycin and have an amplified mdr-like gene. A battery of cell lines was step-wise selected with increasing concentrations of vinblastine and cell lines were analyzed at various points in the selection process. The growth of these cell lines was compared with the parent *L. enriettii* in the presence of increasing concentrations of vinblastine. For example, the IC_{50} (vinblastine) of parental cells was about 30 $\mu g\ ml^{-1}$, while the LeV900 cells grew continuously at vinblastine concentrations of 25–400 $\mu g\ ml^{-1}$ (see Fig. 1A). Previous work with the vinblastine-resistant *L. donovani* strain demonstrated that those cells were cross-resistant to multiple other drugs similar to the observations with multidrug-resistant mammalian cells [18]. To determine if the vinblastine-resistant *L. enriettii* cells were similarly resistant to other drugs, the LeV900 cells were tested for growth in increasing concentrations of puromycin. As can be seen in Fig. 1B, the IC_{50} (puromycin) of the parental cells was about 50 μM, while the LeV900 cells grew at concentrations of 500 μM.

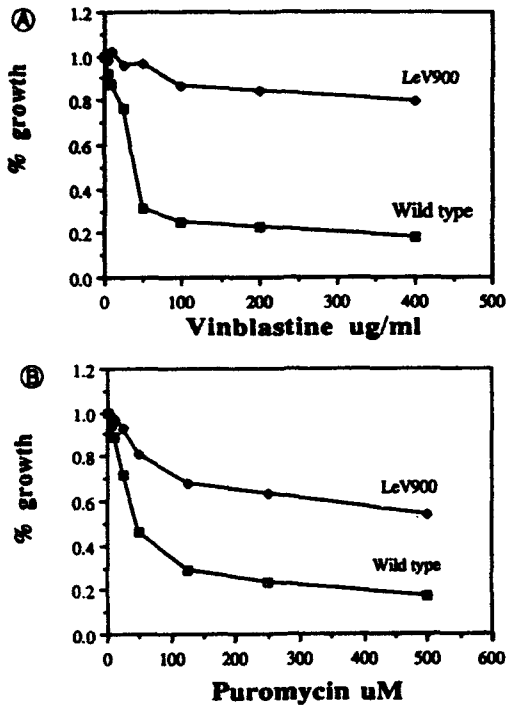


Fig. 1. Comparison of growth of the wild type (Le) and resistant *L. enriettii* cells (LeV900). Le and LeV900 cells were grown at different concentration of drugs: (A) vinblastine; (B) puromycin. The curve was plotted at each drug concentration as a percentage of control growth.

Previous work in mammalian systems and in vinblastine-resistant *L. donovani* demonstrated an amplification of *mdr* genes associated with vinblastine resistance [1,18]. DNA from the wild-type and vinblastine-resistant *L. enriettii* cells were digested with *Bam*HI and Southern blotted. Hybridization with radiolabeled LEMDR06 [18] showed that there was an increased hybridization signal (ranging from 10–20 copies) in the resistant cells when compared to α-tubulin which was used as a control for the amount of DNA loaded onto the gel (Fig. 2). The gene containing LEMDR06 is designated *lemdr1*. Similar results were obtained when the enzymes *Sal*I, *Sac*I and *Cla*I were used (data not shown). Rehybridization of the same blot with a probe derived from another previously characterized *mdr*-like gene in *Leishmania tarentolae*, *ltpgpA* [17], showed that there was no amplification of *ltpgpA* in the resistant cells (data not shown).

Pulsed field gel electrophoresis and γ irradiation of chromosomal DNA blocks from sensitive and resistant L. enriettii cell lines. In *Leishmania* spp., amplified genes frequently occur as extra-

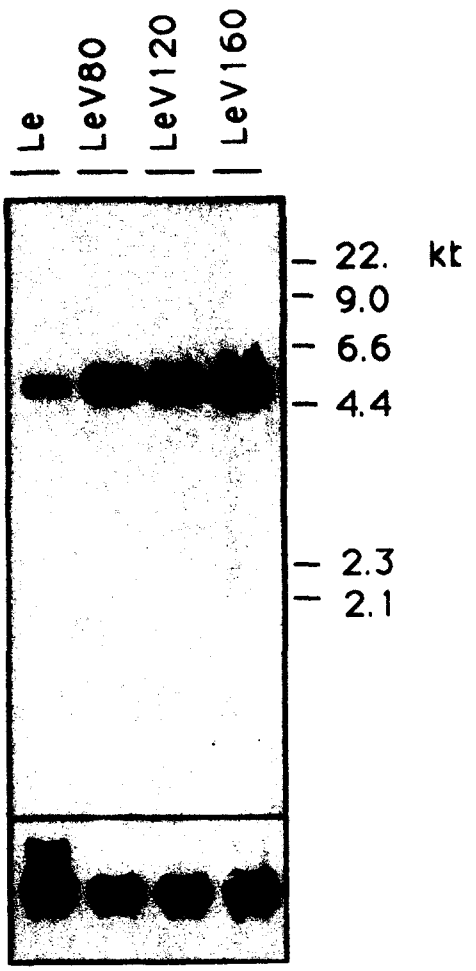


Fig. 2. Southern analysis of genomic DNA from wild-type and resistant *L. enriettii* cells. Genomic DNA (5 μg) was digested with *Bam*HI, fractionated on 0.8% agarose gel, blotted onto nylon paper and hybridized with radiolabeled LEMDR06. In the bottom panel, the same filter was hybridized with α-tubulin probe (as a loading control). Wild type, Le. Resistant cells, LeV80, LeV120, LeV160 which were growing at a vinblastine concentration of 80, 120 and 160 μg ml^{-1}, respectively.

chromosomal circular elements [42–44]. Three experimental methods were used to determine if the amplified *lemdr1* gene was present on an extrachromosomal element, chromosomal gel analysis, γ irradiation, and alkaline lysis purification of extrachromosomal DNA. First, chromosomal DNA preparations were analyzed using TAFE. The results (Fig. 3A and 3B) demonstrated an additional band which hybridized with the *lemdr1* specific probe in chromosomal preparations from vinblastine-resistant cells when compared to those isolated from the sensitive parent. Secondly, the migration of this new element was pulse time independent consistent with a circular structure (Fig. 3C and D) [45,46]. We designated this extra-chromosomal circular element as the V-circle (V stands for Vinblastine). The mobility of the V-circle in LeV80, LeV120, and LeV160 cells remained approximately constant (Fig. 3B and D)

The chromosomal DNA preparations were subjected to γ irradiation before TAFE analysis. Previous work demonstrated that γ irradiation is an effective method of cleaving extrachromosomal circular elements resulting in linear molecules [45,46]. Chromosomal DNA blocks from LeV160 cells were irradiated with increasing amounts of γ irradiation and the migration of the extrachromosomal element was altered from an apparent size of 60 kb to a size of 35–40 kb (Fig. 4). This result was consistent with the double stranded cleavage of a circular element by γ irradiation. Based on this result, the size of the extrachromosomal element, referred to as the V-circle, was estimated to be about 35–40 kb.

Finally, the method of alkaline lysis was used to enrich extrachromosomal elements and the amplified *lemdr1* gene copurified with the extrachromosomal element fraction [47]. Limited DNaseI digestion was performed on the purified DNA. It was found that the circular DNA was digested by the DNaseI to give rise to nicked circular and linear form of DNA in a time-dependent manner (data not shown). This further supports the above suggestion that the *lemdr1* amplicon is an extra-chromosomal circular element.

Restriction mapping of the V-circle and identification of the circle junction. The *lemdr1* gene was localized on 2 overlapping phage clones (λ3 and λ99) encompassing a region of approximately 26 kb. In order to map the structure of the amplicon, different restriction fragments (labeled 1–9 in Fig. 5) from the 2 clones were used to probe the *Bam*HI digested

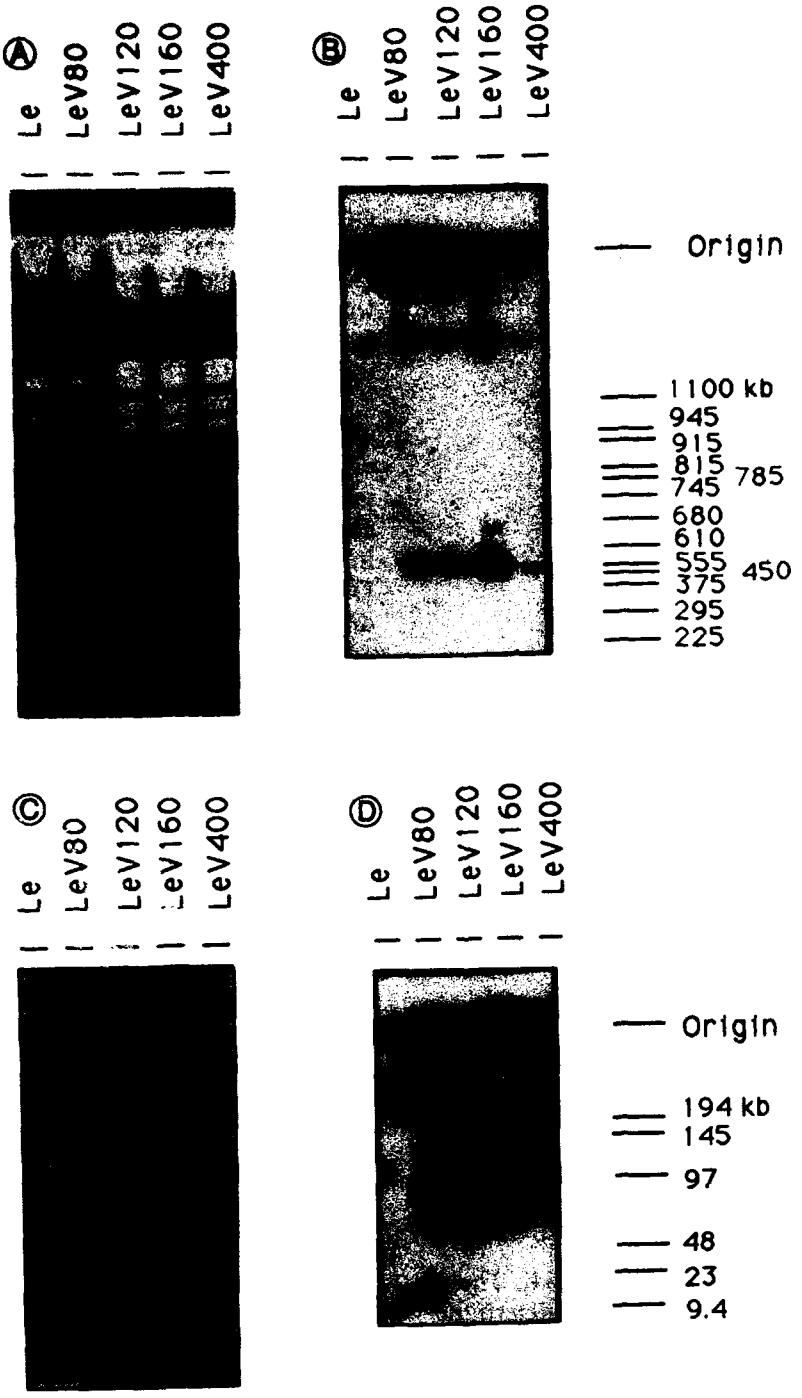


Fig. 3. TAFE of genomic DNA from wild-type and resistant cells. DNA was separated on 1% FastLane agarose by TAFE, blotted onto nylon paper and hybridized with radiolabeled LEMDR06. (A) Ethidium bromide staining of the TAFE using 1 min pulse time. Wild type (Le) and Resistant cells (LeV80, 120, 160 and LeV400). (B) Southern blot of A, hybridized with radiolabeled LEMDR06. (C) Ethidium bromide staining of TAFE using 4 s pulse time; (D) Southern blot of C, hybridized with radiolabeled LEMDR06. Considerable amounts of nicked circular DNA were trapped in the well, as indicated by the strong hybridization signal in the origin.

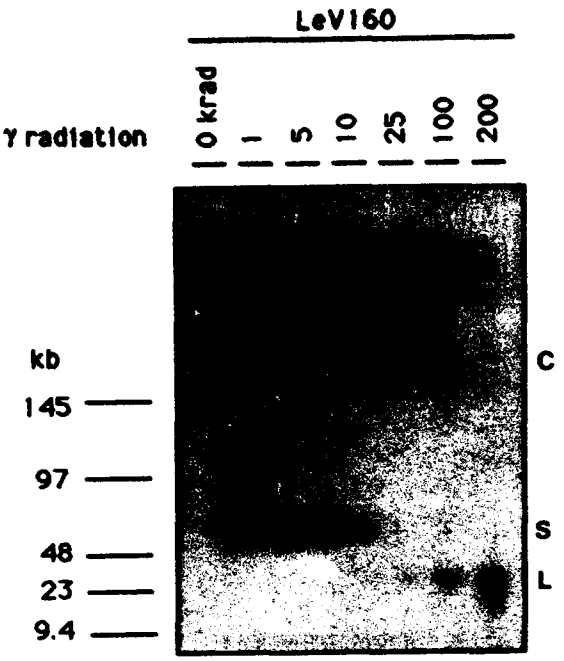


Fig. 4. γ-irradiation of genomic DNA from resistant cells. Agarose blocks containing genomic DNA from LeV160 cells were irradiated with increasing doses of γ ray from 0 to 200 krad. The γ ray was from a source of ^{60}Co. The DNA was then separated on TAFE using a pulse time of 4 s, blotted onto nylon paper, and hybridized with radiolabeled LEMDR06. C, chromosomal copy of *lemdr1*; S, supercoiled form of V-circle; L, linearized form of V-circle.

DNA of both wild-type and resistant cells. It was found that when probes 1–5 were used, there was an increase in signal intensity in the resistant cells compared to the wild type (Fig. 6A and data not shown). The restriction pattern remained the same between the wild type and the resistant cells. However, when the probe 6 was used, the restriction pattern between the wild type and the mutant cells differed. The expected 2.4-kb band was present in the same intensity in both the wild-type and resistant cells. However, there was an extra >15-kb band amplified in the resistant cells (Fig. 6B). It is envisioned that the 2.4-kb *Bam*HI band was derived from the unamplified chromosomal region, while the extra >15-kb band was derived from the amplified extrachromosomal region. Therefore, the novel >15-kb band was created when the circularization occurred within the probe 6 region (the 2.4-kb *Bam*HI fragment). Probe 6 also detected new amplified fragments in the *Sal*I, *Sac*I and *Cla*I digested genomic DNA (data not shown). When probes 7–10 were used, there were no changes in either the signal intensity or the restriction pattern, indicating that this region of genomic DNA was not amplified (Fig. 6C and data not shown). We hypothesize that the recombination event which results in the formation of the V circle occurs at a site within the 2.4-kb *Bam*HI fragment.

Sequence of lemdr1. The region encompassing the putative coding region of the *lemdr1* gene was sequenced, and the longest open reading frame (ORF) found was 1280 amino acids (Fig. 7). A protein of a size of about 140 kDa is predicted. A hydropathy plot [41] revealed that it has 12 transmembrane domains and 2 hydrophilic putative nucleotide binding sites symmetrically divided into 2 halves (Fig. 7). These structures are very similar to other *mdr* gene products [1]. Comparison of the *lemdr1* sequence with the GenBank™ database revealed that the *lemdr1* has a 37% sequence identity with the human *mdr1* gene, at the amino acid level, with the greatest identity found in the putative ATP binding domain. When compared with the *L. donovani ldmdr1* gene [54], there is an 83% sequence identity at the amino acid level. The major difference between the predicted proteins for *lemdr1* and *ldmdr1* is in the choice of initiation codon. The putative *lemdr1* start codon has a counterpart in the *ldmdr1*, but the longest ORF of *ldmdr1* predicts an AUG start codon 61 amino acids upstream and in-frame of the putative AUG start codon of *lemdr1*. In addition, the comparison of the predicted protein sequences for *lemdr1* and *ldmdr1* shows the highest divergence in the first 40 amino acids of the *lemdr1* protein.

No data are available concerning post-translational modification of the predicted *lemdr1* gene product, however, a comparison of putative glycosylation sites indicated that the three *N*-glycosylation sites of human *mdr1* are absent in the *lemdr1* [1]. All potential *N*-glycosylation sites of *lemdr1* are either buried

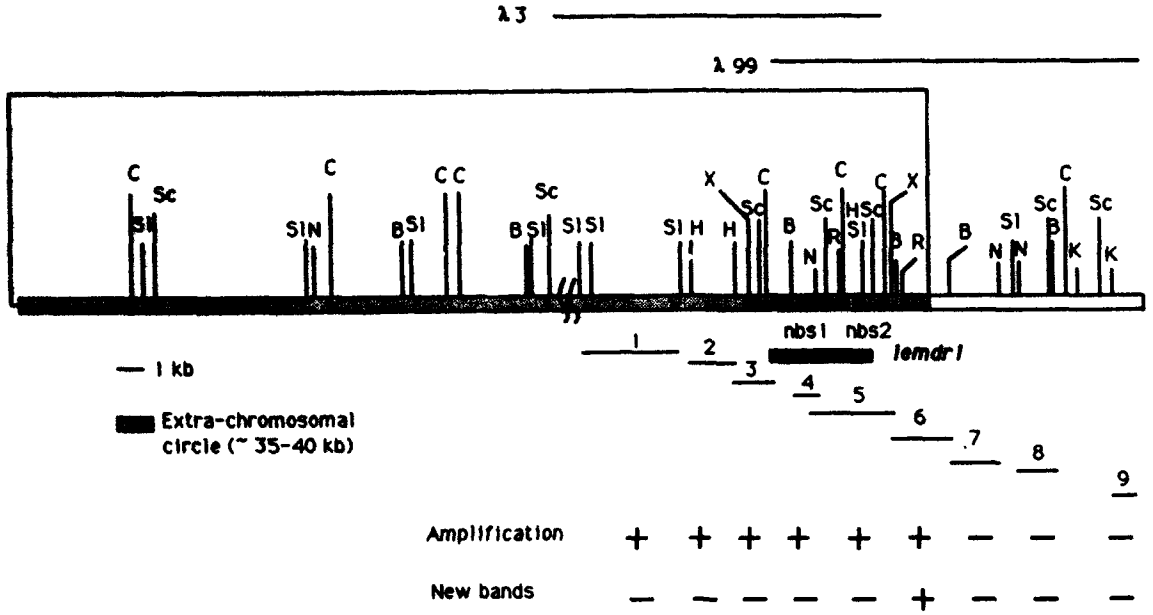


Fig. 5. Restriction map of λ3, λ99 and the V-circle. Regions covered by λ3 and λ99 were represented as 2 bars on top of the diagram. The 2 nucleotide binding sites of *lemdr1* are represented as black boxes (nbs1 and nbs2). Coding region of *lemdr1* is represented by a dark bar underneath the restriction map. The stippled region represents the region which is amplified as extra-chromosomal circle. Fragments 1–9 were used as probes for hybridization analysis. Sc, *Sac*I; Sl, *Sal*I; B, *Bam*HI; R, *Eco*RI; K, *Kpn*I; N, *Not*I; C, *Cla*I; X, *Xho*I; H, *Hin*cII. + and − signs indicate the presence or absence of amplification or new bands (see Fig. 6) in the resistant cells.

in the predicted transmembrane domains or in the putative cytoplasmic side of the protein. Similarly, no potential *N*-glycosylation sites of *ldmdr1* are present on the putative extracellular side of the protein [54].

Transfection with the lemdr1 gene confers drug resistance. In order to show that the *lemdr1* gene alone can confer drug resistance to sensitive *Leishmania* cells, a 7-kb *Xho*I fragment containing the *lemdr1* coding sequence was cloned into the expression vector pALTNEO [33] for transfection analysis. The construct was designated as pNEOU2 (Fig. 8A). Stable transfected cell lines established from this construct were approximately 3-fold more resistant to vinblastine than cell lines transfected with the control vector pALTNEO (Fig. 8B). However, the level of resistance is lower than the drug-resistant cell line LeV160 which was established by step-wise drug selection (Fig. 8B). The copy number of the *lemdr1* gene in the pNEOU2 cell line is >20 (data not shown) which is higher than that found in LeV160 (10–20 copies). Analysis of the Northern blot of pNEOU2 and LeV160 also revealed a higher level of *lemdr1* mRNA in the pNEOU2 (data not shown). This result demonstrates a role for the *lemdr1* gene in vinblastine resistance; however, other additional factors may contribute to high level vinblastine resistance.

Discussion

Here we report the identification, cloning and sequencing of an *mdr*-like gene from *L. enriettii*, *lemdr1*, and demonstrate that this gene is amplified on an extrachromosomal circle of about 35–40 kb in vinblastine-resistant *L. enriettii*. Functional analysis of the *lemdr1* gene by transfection of wild-type cells demonstrated that expression of the *lemdr1* gene conferred vinblastine resistance to wild-type cells. The level of resistance was lower than

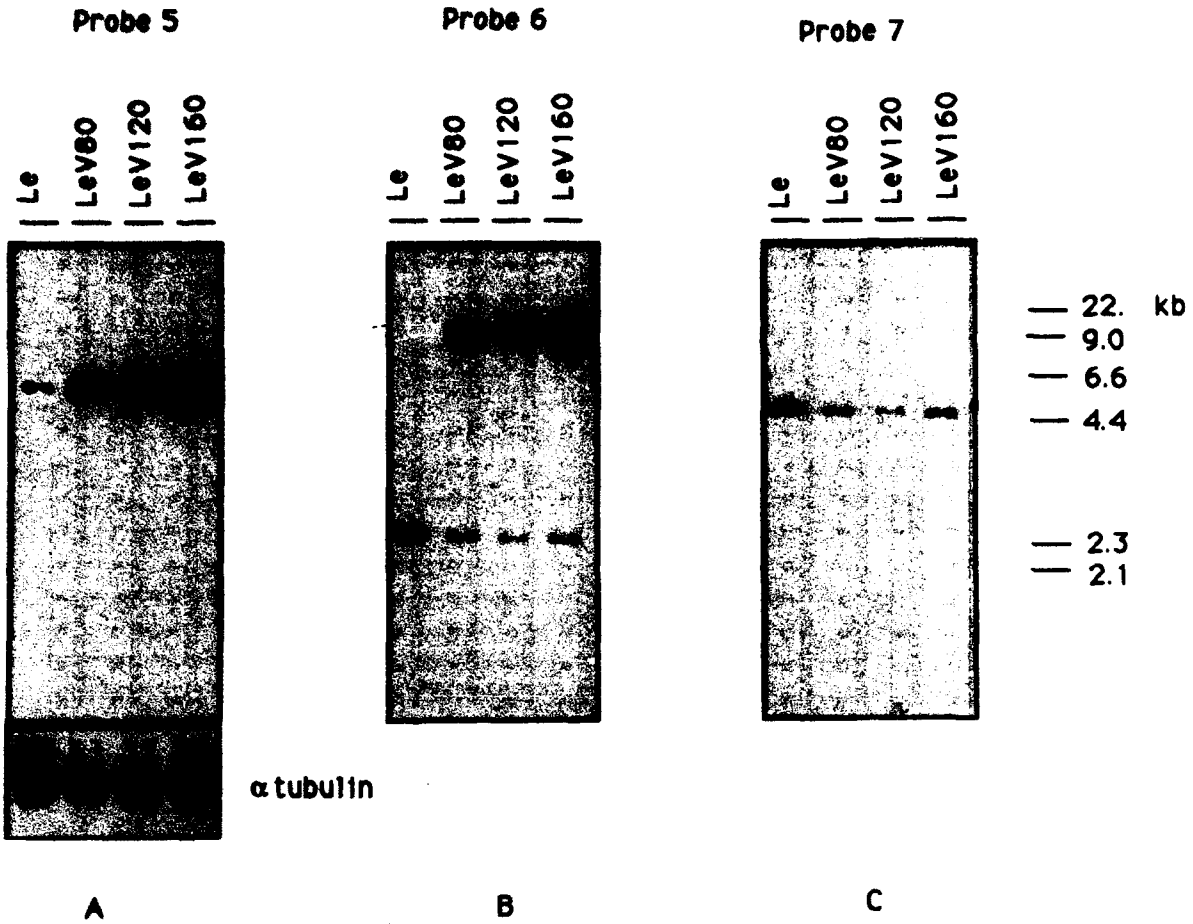
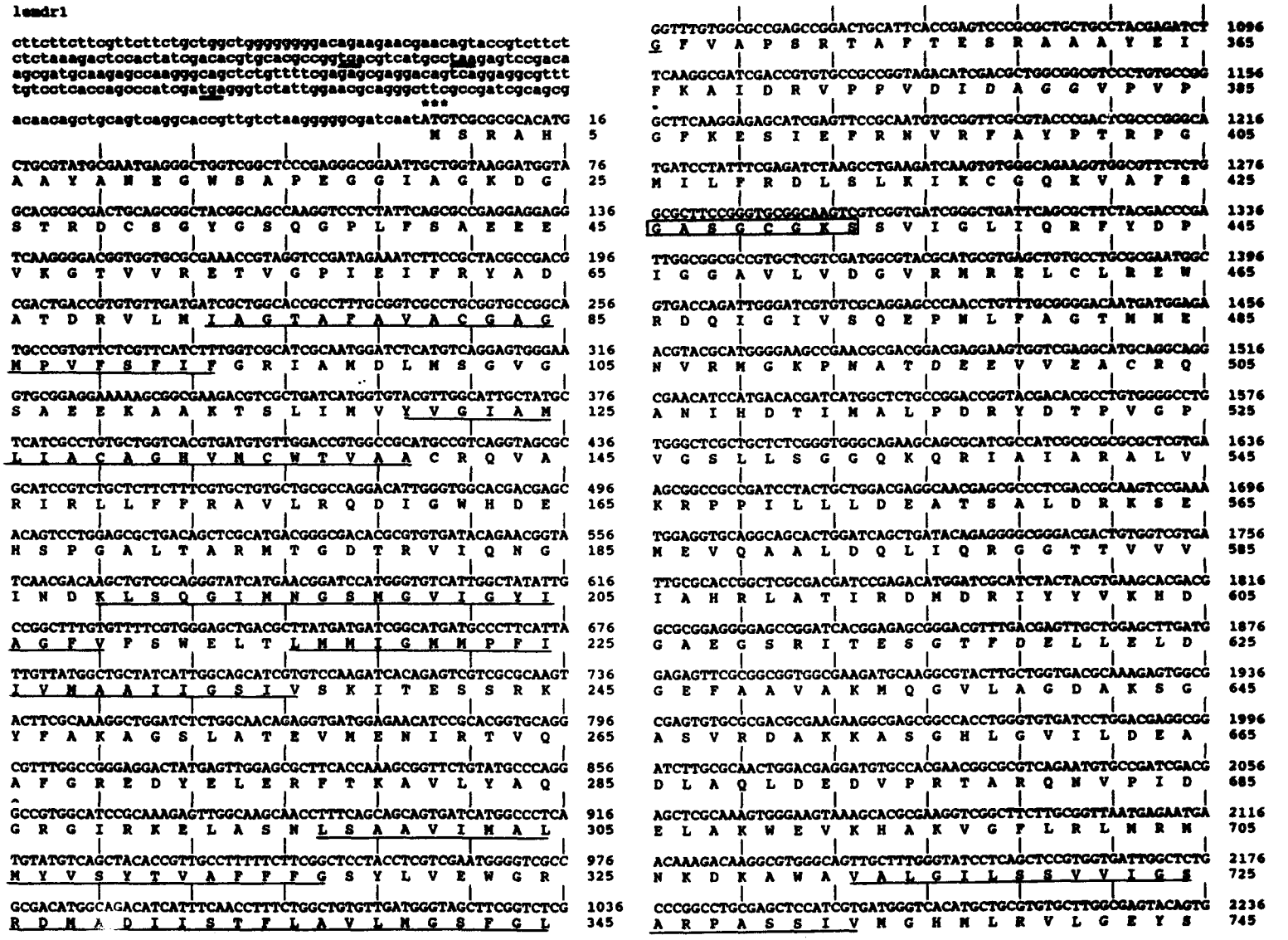


Fig. 6. Southern analysis of genomic DNA from wild-type and resistant cells. Genomic DNA (5 μg) from wild-type and resistant cells (LeV80, Le V120 and LeV160) were digested with *Bam*HI, separated on 0.8% agarose gel, blotted onto nylon paper and hybridized with probes 5–7 (see Fig. 5). The same filter was stripped with 0.4 N NaOH before rehybridized with another probe. The bottom panel shows hybridization with the α-tubulin probe as a loading control.

that observed with the step-wise selected mutant cells. The longest open reading frame in the cloned gene is 1280 amino acids with a predicted protein of 140 kDa. The predicted protein has a structure similar to that for all other reported P-glycoproteins, namely 12 transmembrane domains and 2 ATP binding sites, arranged in 2 similar half-molecules. Comparison of the primary amino acid sequence with other known *mdr* genes demonstrates a significant homology with 37% amino acid identity with human *mdr1* and 83% identity with the *L. donovani*, *ldmdr1* gene. The correlation between the vinblastine resistance and the over-expression of an *mdr* gene was first observed in the mammalian cells. Therefore, this phenomenon was highly conserved and reproducible ranging from the higher to the lower eukaryotes.

Previously, another *mdr* homologue (*ltpgpA*) has been cloned in *L. tarentolae* [17]. Comparison of all 3 *mdr* homologues showed that *lemdr1* is 83% and 24% identical to *ldmdr1* and *ltpgpA*, respectively, at the amino acid level. The sequence of *lemdr1* showed a 37% identity to either mouse *mdr2* or human *mdr1*, at the amino acid level, while the *ltpgpA* only showed a 22% identity to the mouse and


lemdr1

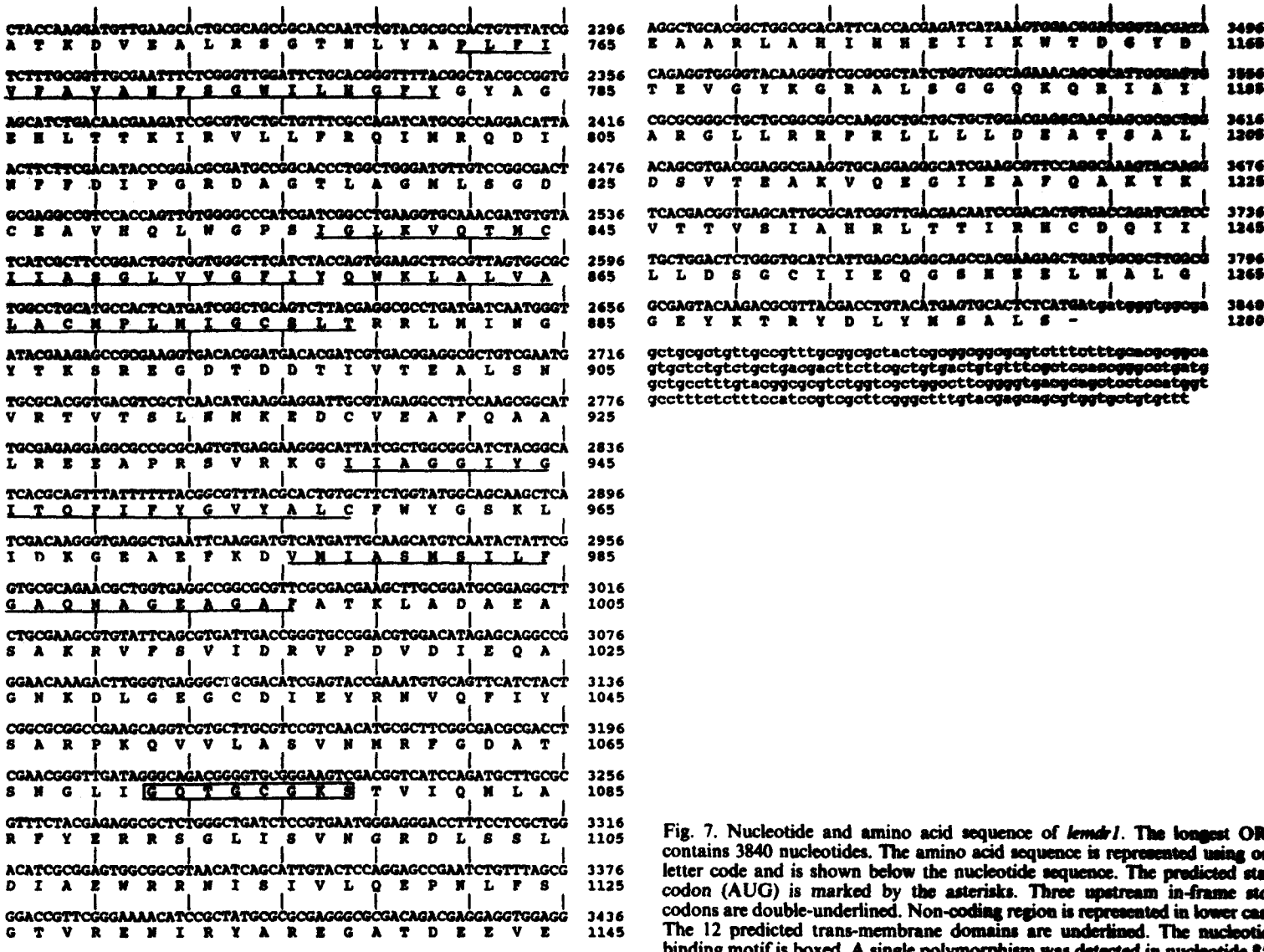


Fig. 7. Nucleotide and amino acid sequence of *lemdr1*. The longest ORF contains 3840 nucleotides. The amino acid sequence is represented using one letter code and is shown below the nucleotide sequence. The predicted start codon (AUG) is marked by the asterisks. Three upstream in-frame stop codons are double-underlined. Non-coding region is represented in lower case. The 12 predicted trans-membrane domains are underlined. The nucleotide binding motif is boxed. A single polymorphism was detected in nucleotide 857 (*) resulting in a predicted amino acid change (position 286) from glycine to aspartic acid.

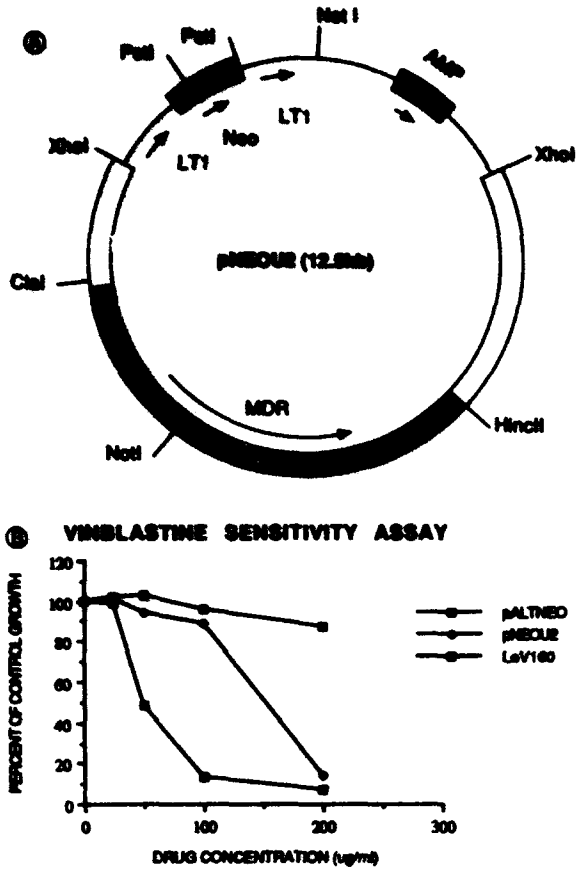


Fig. 8. Transfection of wild-type *L. enriettii* with *lemdr1* confers vinblastine resistance. (*A*) Restriction map of pNEOU2. It was prepared by subcloning the 7-kb *Xho*I fragment (see Fig. 5) into pALTNEO. (B) Stably transfected cell lines of pALTNEO and pNEOU2 and the positive control LeV160 were assayed for vinblastine sensitivity at 0, 25, 50, 75, 100, and 200 $\mu g\ ml^{-1}$.

human genes. This suggested that the *lemdr1* and *ldmdr1* genes are more closely related to the mammalian *mdr* genes than is *ltpgpA*.

Functionally, the *lemdr1* gene and the *ltpgpA* are also different. Transfection experiments showed that the *ltpgpA* homologue in *L. major*, *lmpgpA*, conferred antimony and arsenite resistance, but not resistance to other hydrophobic drugs like vinblastine. On the contrary, the over-expression of *ldmdr1* in transfected *L. donovani* cell line can confer MDR phenotype (18). This work has further confirmed that the *lemdr1* gene is involved in mediating vinblastine resistance in *Leishmania enriettii*.

The level of drug resistance does not correlate with the gene copy number of *lemdr1* in these transfection experiments. There are several possible explanations including sequence polymorphism as recently demonstrated for the *ldmdr1* gene [54], differences in RNA processing signals in the transfected gene as compared to the gene on the amplified circle and finally, the role of other genes or sequences in the amplified V-circle.

The *lemdr1* multidrug resistance gene is amplified as an extra-chromosomal circle (V-circle). This is different from the *mdr* gene amplification in mammalian cells, which is associated with either large circular double minutes or with long chromosomally integrated homogeneously staining regions (1). In *Leishmania*, there are previous examples of extra-chromosomal circles associated with drug resistance, including the R-circle (containing *dhfr-ts*) [43], H-circle (containing *ltpgpA* or *lmpgpA* and *ltdh* or *hmtx*^{r}) [17,50,51], a 63-kb circle (containing the *N*-acetyl-glucosamine-1-phosphate transferase gene) [52], and a 68-kb circle of unknown function in difluoromethylornithine-resistant cells [53].

These results taken together with those in *L. donovani* indicate that the *mdr*-like genes, *lemdr1* and *ldmdr1* are involved in conferring a multidrug resistance phenotype in *Leishmania* sp. similar to that originally observed in mammalian neoplastic cells. The role this mechanism of drug resistance plays in clinical disease is unknown, however, the relative ease with which resistant mutants were isolated in 2 different species indicates that such mechanisms could play a role in clinical resistance either now or in the future.

Acknowledgements

We would like to thank Drs. Mark Rodgers, James Tobin and Maria Curotto de Lafaille for helpful discussions; Ramona Gonski for manuscript preparation and Dr. Mark Ouell-

ette for providing the *ltpgpA* probe.

This work was supported by grants from the John D. and Catherine T. MacArthur Foundation and the NIH (AI-21365 and AI-27872) to D.F.W. A.K.C.W. is funded by a postdoctoral fellowship from the NSERC of Canada. During the early phase of this work D.F.W. was a Burroughs Wellcome Scholar in Molecular Parasitology.

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REPRINTED FROM:

Molecular and Biochemical Parasitology

Molecular and Biochemical Parasitology, 62 (1993) 83–92
 / 0166-6851/93/$06.00

MOLBIO 02065

Characterization of the *pfmdr2* gene for *Plasmodium falciparum*

Mariano G. Zalis, Craig M. Wilson[1], Yun Zhang and Dyann F. Wirth*

Department of Tropical Public Health, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115, USA

(Received 31 March 1993; accepted 7 August 1993)

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Molecular and Biochemical Parasitology

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The journal provides a medium for the rapid publication of investigations of the molecular biology, molecular immunology and biochemistry of parasitic protozoa and helminths and their interactions with both the definitive and intermediate host. The main subject areas covered are: chemical structure, biosynthesis, degradation, properties and function of small molecular weight substances, DNA, RNA, proteins, lipids and carbohydrates – intermediary metabolism and bioenergetics – molecular and biochemical studies on the mode of action of antiparasitic drugs – molecular and biochemical aspects of membrane structure and function – molecular and biochemical aspects of host–parasite relationships including analysis of parasitic escape mechanisms – characterisation of parasite antigen and parasite and host cell surface receptors – characterisation of genes by biophysical and biochemical methods, including recombinant DNA technology – analysis of gene structure, function and expression – mechanisms of genetic recombination.

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We report the characterization of the *pfmdr2* gene which is a gene related to the P-glycoprotein family but with a somewhat different structure than the mdr genes. Based on DNA sequence analysis of genomic clones, we have discovered that the *pfmdr2* gene has 10 predicted transmembrane domains and a single ATP-binding site. In a homology search using GenBank sequences, we discovered that the *pfmdr2* gene has a significant homology with the *hmt1* gene in yeast. The yeast *hmt1* gene is involved in cadmium resistance and is hypothesized to transport cadmium containing complexes from the cell. We have further characterized the *pfmdr2* gene expression by northern analysis and discovered that it is expressed in a stage-specific manner, only at the trophozoite stage and not in ring stages. We have prepared a rabbit antibody to a recombinant fusion protein expressing a portion of the *pfmdr2* coding region. In IFA analysis, this antibody stains trophozoites and not ring stages. Western analysis reveals a protein of approximately 110 kDa which is consistent with the size of the predicted open reading frame based on DNA sequence analysis. Based on this analysis and previous work, there is no evidence for a change in *pfmdr2* expression in drug-resistant versus drug-sensitive parasites.

Key words: *Plasmodium falciparum*; *pfmdr2* gene; Stage-specific expression

Introduction

Malaria remains a major disease threat throughout the tropical world. Malaria control programs have failed to limit the spread of disease and the emergence of parasites resistant to drugs has increased the severity of the problem [1]. In an attempt to develop new means to combat this emergence of resistance, we have begun a molecular analysis of drug resistance. This work has focused on a family of proteins related to the mammalian multidrug resistance (mdr) genes.

The mdr genes are members of a superfamily of genes that are similar in sequence, predicted secondary structure and function referred to as the ATP-binding cassette (ABC) proteins [2]. They have been identified in such evolutionarily diverse organism as bacteria [3], yeast [4], protozoa [5] and mammals with a putative ATP. The transport substrates of these gene products are extremely varied in nature and size ranging from small molecules to peptides and polypeptides [6–10]. The ABC superfamily homologs have been also identified in protozoan parasites including *Plasmodium falciparum* [11,12], *Leishmania tarantulae* [5] and *Entamoeba histolytica* [13]. Two genes have been identified in *Plasmodium falciparum* which have sequence homology with the ABC family [11,12]. One of these, *pfmdr1* has a similar structure with 12 predicted transmembrane domains and 2 ATP binding sites and is

*Corresponding author. Tel: (617) 432-1563; Fax: (617) 738-4914.

[1] *Present address:* Division of Geographic Medicine, University of Alabama at Birmingham, Birmingham, AL, USA.

Abbreviations: MDR, multiple-drug resistance; ABC, ATP-binding cassette; PCR, polymerase chain reaction; IFA, immunofluorescence assay.

Note: Nucleotide sequence data reported in this paper have been submitted to the GenBank™ data base with the accession number L13381.

implicated in mefloquine resistance [11,23,32–33]. We now report the structure and characterization of the *pfmdr2* gene.

Materials and Methods

Parasites. The *P. falciparum* strains HB3 [14] from Honduras I\CDC, W2 and W2mef from Indochina [15] were maintained in vitro according to a modification of the method of Trager and Jensen [16]. The isolates were maintained in 5% suspension of type A+ erythrocytes in RPMI 1640 supplemented with 32 mM $NaHCO_3$/ 12 mM TES/ 37 mM hypoxanthine/ 2 mM glutamine/ 10 mM glucose/ 10% human plasma in an atmosphere of 1% O_2/ 5% CO_2/ 94% nitrogen. The *pfmdr2* gene was characterized using both the HB3 and W2 *P. falciparum* clones.

P. falciparum (HB3 strain) genomic DNA library. A genomic library was constructed using HB3 genomic DNA which was partially digested with *Apo*I (New England Biolabs, MA), and ligated into the *Eco*RI site of LambdaZap (Stratagene, CA). Genomic clones were selected from an amplified library using *pfmdr2* specific probes generated by polymerase Chain Reaction (PCR) (probe 1: nucleotides: 10–320 of the coding region and nucleotides: 2308–2839 of the coding region). The selected phage clones were converted to plasmids by using the manufacturer's protocol. Double-stranded DNA was sequenced in both directions [17].

Inverse PCR. The method described by Triglia et al. allows the amplification of flanking regions of known DNA sequences by PCR [18]. W2 DNA was digested with *Sau*3AI, *Taq*I or *Hpa*II (New England Biolabs) and analyzed by Southern blot using a 450-bp PCR product corresponding to the *pfmdr2* nucleotide binding site [12]. The ligation reaction contained 500 ng of DNA/ 1.2 U μl^{-1} of T4 Ligase (N.E. Biolabs)/ 1 mM of ATP/ 30 mM Tris-HCl pH 7.8/ 10 mM $MgCl_2$/ 10 mM DTT in a total volume of 200 μl. The ligation reaction was precipitated with ethanol. The circularized DNA was amplified by PCR with the use of a number of primers. The 0.75 kb *Sau*3AI DNA fragment was amplified by 5'-GGATCCTATCTTGTGGTACTA-3'(sense) which introduces a *Bam*HI site, and 5'-AAGCTTACAAGCTGTTAAATCG-3' (antisense), which introduces a *Hin*dIII site. The 0.5-kb *Taq*I fragment was amplified by 5'-GGAAAATTAGATGCCACTGAG-3'(sense) and 5'-GGTATCTTGTGGTACTATACC-3' (antisense). The 1.5-kb *Taq*I fragment was amplified by 5'ATGTACATTTTAATTACCCAA (sense) and 5'-AGTAAATGATTTAATAATAGTA-3' (antisense). The primers 5'-GATATGAATATATCATATA-3' (sense) and 5'-TTATCTGTTCCTAATAAGC-3' (antisense) were used to amplify the 0.8-kb *Hpa*II fragment. The PCR was carried out under the following conditions: 100 ng of DNA as template, 30 pmol of each primer, 0.5 U of *Taq* polymerase (Cetus), 70 mM Tris-HCl pH 8.8, 20 mM NH_2SO_4, 2 mM $MgCl_2$, 1 mM DTT, 100 $\mu g\ ml^{-1}$ BSA, 0.1% Triton X-100 in a 100 μl reaction for 30 cycles of 94°C × 1 min, 47°C × 3 min, and 72°C × 12 min. The PCR products were purified on a 1% low-melting agarose gel digested with appropriate enzymes and cloned into pBluscript vector (Stratagene) at *Bam*HI-*Hin*dIII restriction sites for the product derived from the *Sau*3AI fragment (clone pf750) and *Eco*RV site for the products amplified from the *Taq*I (clones pf1.5 and pf500) and *Hpa*II (clone pf800) fragments.

Sequence analysis. Plasmids containing *pfmdr2* gene fragments were sequenced by using a modification of the dideoxynucleotide chain termination [17] method with Sequenase enzyme (USB) and *Taq* polymerase (Promega). All the sequences were determined on both strands using specific oligonucleotide primers. The newly determined *pfmdr2* DNA sequence was analyzed using the PCGENE software (IntelliGenetics, Inc.) and the deduced amino acid sequence was compared in the GenBank sequences. The predicted membrane spanning regions of the *pfmdr2* protein sequence were analyzed using the HELIXMEM program

algorithms of Eisenberg et al. [19] in the PCGENE software.

Southern analysis. DNA from *P. falciparum* was extracted [23], digested (1 μg/lane) with *Eco*RI and resolved on 1% agarose gels and transferred to nylon membranes. The hybridization was performed using PCR generated fragments of the *pfmdr2* gene corresponding to bases 10–357 of the coding region simultaneously with the Circumsporozoite gene [28]. The DNA fragments were radiolabeled using random oligonucleotide priming technique [20].

Northern analysis. Total RNA from HB3, W2 and W2mef was extracted as described by Volkmann et al. [21]. RNA from stage-specific parasites was isolated after synchronization with 5% sorbitol [24] followed by incubation with aphidocholine (1.5 μg ml^{-1} final) [25]. Poly(A)$^+$ RNA was isolated from total mixed stage RNA using a oligo(dT) column as described by the manufacturer (Pharmacia).

Total RNA (10 μg) and Poly(A)$^+$ RNA were fractionated on a 1.3% agarose gel with 6.6% of formaldehyde and transferred to nitrocellulose filter. The hybridization was performed using the *pfmdr2* probe (clone pf750) in 1% SDS/ 1 M NaCl/ 10% dextran sulfate and 50% formamide at 42°C overnight.

Construction of the fusion protein. pfmdr2 sequences contained in the transmembrane domain were prepared by PCR from W2 genomic DNA. The sense primer contained the sequence 5'-CGGGGATCCGATATATGAATATATCATAA-3' which introduces a *Bam*HI. The anti-sense primer contained the sequences 5'-ATGCAAGCTTTCATATCTATCCTTTTGC-3' which introduces a *Hin*dIII site. After PCR, the 400-bp product was digested with *Bam*HI and *Hin*dIII.The fragment was cloned into the *Bam*HI-*Hin*dIII site of the pUR291 vector [26] and transformed into *Escherichia coli* XL1-Blue strain (Stratagene). The constructions were sequenced (as described above) to ensure that the correct reading frame was maintained. The fusion protein (β-galactosidase-*pfmdr2*) was expressed in the bacteria by IPTG (1 mM final) induction for 3 h at 37°C.

The bacterial culture was then centrifuged and resuspended in 0.5 ml of lysis buffer (50 mM Tris-HCl pH 8.0/ 1 mM EDTA/ 100 mM NaCl/ 0.5% Triton X-100/ 1 mM leupeptine/ 1 mM PMSF) and incubated in room temperature for 30 min. The suspension was centrifuged 12 000 × *g* and the supernatant was kept at −70°C until used. The solubilized bacterial proteins were separated in 8% SDS-PAGE and the band corresponding to the fusion protein was cut out of the gel. To immunize the rabbits, the gel slice was homogenized with 2 ml of PBS and then mixed with 1 vol. of either complete Freund's adjuvant (CFA) of incomplete Freund's adjuvant (IFA). For each immunization or boost, approximately 1 mg of protein was used. Injections were done at multiple subcutaneous sites according to the following schedule: day 1, immunize with CFA; days 15 and 22, boost with IFA; day 30, bleed rabbits and test serum by indirect immunofluorescence.

Western blot analysis. Parasitized red blood cells (10%–15% parasitemia) were pelleted and washed 3 times with Trager's buffer (60 mM NaCl; 60 mM KCl, 10 mM Dextrose, 10 mM $NaHCO_3$, 1 mM NaH_2PO_4; pH 7.8). The washed cells were suspended at the same buffer to 25% hematocrit and PMSF was added to a final concentration of 1 mM. The red cells were lysed by adding, while vortexing, one tenth volume of a saponin-saline solution (0.165% saponin and 0.85% NaCl in H_2O). The resulting parasite pellet was washed 3 times with cold Trager's buffer containing PMSF and stored at −20°C.

The parasites proteins were solubilized in 50 mM Tris-HCl pH 6.8 containing 2% SDS. The solubilized proteins were separated by 8% SDS-PAGE [22]. Proteins were assayed by bicinchonic acid (BCA) protein assay method using BCA protein assay reagents (Pierce). The separated proteins were electrophoretically transferred to a Immobilon-P membrane (Millipore) and incubated in PBS containing

0,3% Tween-20 and non-fat milk. The rabbit antiserum against the *pfmdr2* fusion protein and its pre-immune serum were incubated at dilution 1/200, with the membranes in the same buffer. The bound antibodies were detected by gold conjugated goat anti-rabbit IgG (BIORAD), which was developed by a gold enhancement kit (BIORAD).

Immunofluorescence. Parasitized erythrocytes were washed twice with PBS, pH 7.3 and used for preparation of thin blood films by cytocentrifugation. After air drying, the slides were fixed in acetone at −20°C for 20 min and stored at −70°C until use. The slides with the parasites were reacted with total rabbit anti-*pfmdr2* sera or preimmune sera at dilution 1/50 in PBS for 60 min at room temperature, washed with PBS, and incubated with FITC-conjugated anti rabbit IgG antibodies. The slides were washed with PBS and mounted in 90% glycerol in PBS containing p-phenylnelediamine. Photographs were performed under UV or transmitted illumination at ×1000 magnification with Fuji color 400 film (Fuji) and by a confocal microscope (Noram Instruments) at ×1000 or ×4000 electronic magnification using a digital color printer (Cannon Co.).

Probes. The hybridizations were performed using different *pfmdr2* probes: The 0.75-kb fragment generated by Inverse PCR (clone pf750), which corresponds to the ATP-binding site region, a fragment generated by polymerase chain reaction (PCR) using the primers 5′-TCAAATTACGAGTATTTAAGATCC-3′ (sense) and 5′-TTCAATATAACTAAGATACCG-3′ (antisense), (nucleotides 10–321) and another fragment using the primers 5′-GTTGGTGATAAAGGAGTC-3′ (sense) and 5′-AAGTATGACATCATTATTTTTGCT-3′ (antisense) (nucleotides 2308–2839). The PCR was carried with the following conditions: 100 ng of W2 genomic DNA as template, 30 pmol of each primer, 0.5 U of *Taq* polymerase (Cetus), 70 mM Tris-HCl pH.8, 20 mM NH_2SO_4, 1 mM $MgCl_2$, 1 mM DTT, 100 $\mu g\ ml^{-1}$ BSA, 0.1% Triton X-100 in a 100 μl reaction for 30 cycles of 94°C × 1 min, 50°C × 3 min, and 72°C × 5 min. The PCR products were purified from 1% low-melting agarose in 1 × TAE. The probes for the *pfmdr1* gene and for the circumsporozoite protein (CSP) gene were described elsewhere [21, 28]. The DNA fragments were radiolabeled using random oligonucleotide priming technique [20].

Results

The pfmdr2 gene is present and stage-specifically expressed in both resistant and sensitive parasites. Several different parasite strains were analyzed for the presence of the *pfmdr2* gene sequence (Fig. 1). Southern analysis demonstrated that in all the strains examined regardless of the sensitivity to various antimalarial drugs, the *pfmdr2* gene was present as a single copy gene with no change in gene copy number. The expression of the *pfmdr2* gene was examined by Northern analysis. Analysis of total mRNA isolated from asynchronous parasite cultures demonstrated the presence of

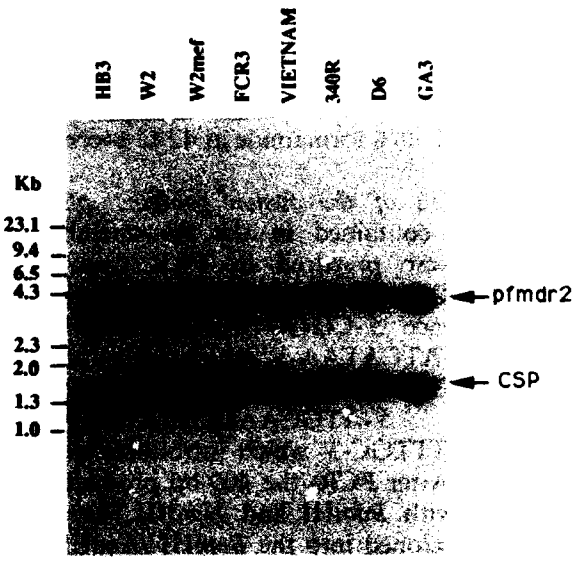

Fig. 1. Quantitative Southern analysis of the *pfmdr2* gene from different isolates of *P. falciparum*. Shown is a representative autoradiogram of a Southern blot of genomic DNA (1 μg/lane). The blot was simultaneously probed with a *pfmdr2* probe (nucleotides: 10–327) and a probe for the CSP gene [28].

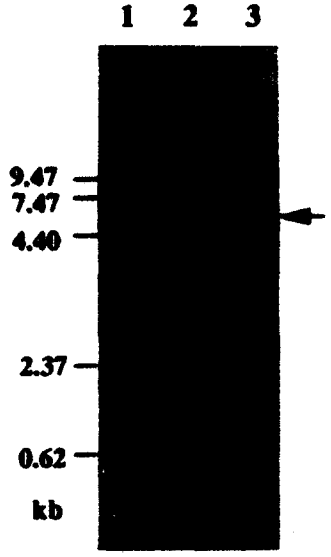

Fig. 2. Oligo analysis of the *pfmdr2* transcript. Poly(A)$^+$ RNA was purified using an oligo column. Total unfractionated W2 RNA (lane 1), oligo(dt)-unbound fraction (lane 2), or oligo bound fraction (lane 3) were resolved on a 1.3% agarose gel in 6.6% formaldehyde. The nucleic acid was transferred to nitrocellulose paper, and hybridization was performed with a *pfmdr2* probe (clone pf750: see Materials and Methods). The arrow indicates the *pfmdr2* transcript. The unbound fraction contains DNA.

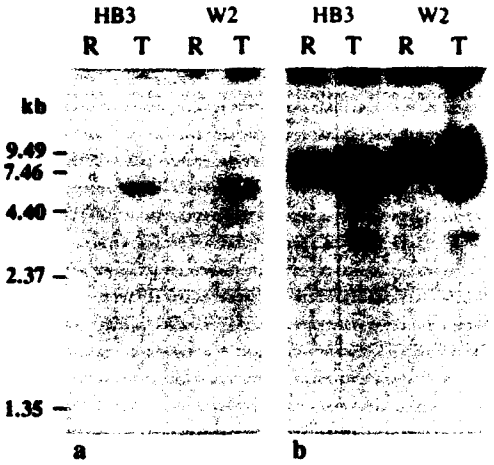

Fig. 3. Analysis of the *pfmdr2* RNA expression in synchronized parasites. W2 and HB3 parasites were synchronized with 5% sorbitol and RNA was purified from the ring stage (R) and trophozoite stage (T) as described in Materials and Methods. The blot was probed with a *pfmdr2* probe (clone pf750) (a). The *pfmdr1* probe (PCR generated fragment; nucleotides 510–1480 of the *pfmdr1* coding region) was used as a parasite stage-specific control, which identified one transcript (8.5 kb) in rings and two transcripts (8.5 and 7.5 kb) in trophozoites [21] (b). There is hybridization to a band >8.5 kb which is DNA.

a single 5.2-kb mRNA with homology to the *pfmdr2* gene. This mRNA is polyadenylated as demonstrated by its binding to oligo(dT) (Fig. 2). No correlation between the expression of the *pfmdr2* gene and drug resistance could be found in several of the drug-resistant and drug-sensitive *P. falciparum* strains analyzed. This is in contrast to the correlation of high expression of the *pfmdr1* mRNA and mefloquine resistance [21]. It is interesting to note that the *pfmdr2* mRNA is significantly smaller than those mRNAs associated with the *pfmdr1* gene [21]. When mRNA from synchronized parasites was examined by Northern analysis, we discovered that the *pfmdr2* mRNA was stage-specifically expressed at the trophozoite stage (Fig. 3). There was no mRNA detectable in ring stage parasites. This is in contrast to *pfmdr1* which is expressed at both the ring and trophozoite stages, but with different sized mRNAs (Fig. 3 and ref. 21).

Sequence of the pfmdr2 gene demonstrates a unique structure. The complete characterization of the *pfmdr2* gene was accomplished by a combination of inverse PCR cloning and analysis of overlapping DNA fragments cloned in λ phage. Analysis of the sequence remitted in the identification of a single open reading frame of 2841 nucleotides (see Fig. 4) with a predicted translation of 947 amino acids. DNA from two different parasite strains, W2 and HB3, was sequenced and the nucleotide sequence is identical within the coding region. The *pfmdr2* amino acid sequence was further analyzed for the presence of potential transmembrane domain using the algorithm of Eisenberg et al. [19], which can distinguish membrane-spanning region from hydrophobic core of globular protein. This analysis has predicted the presence of 10 transmembrane domains in the hydrophobic portion of the sequence and a single hydro-

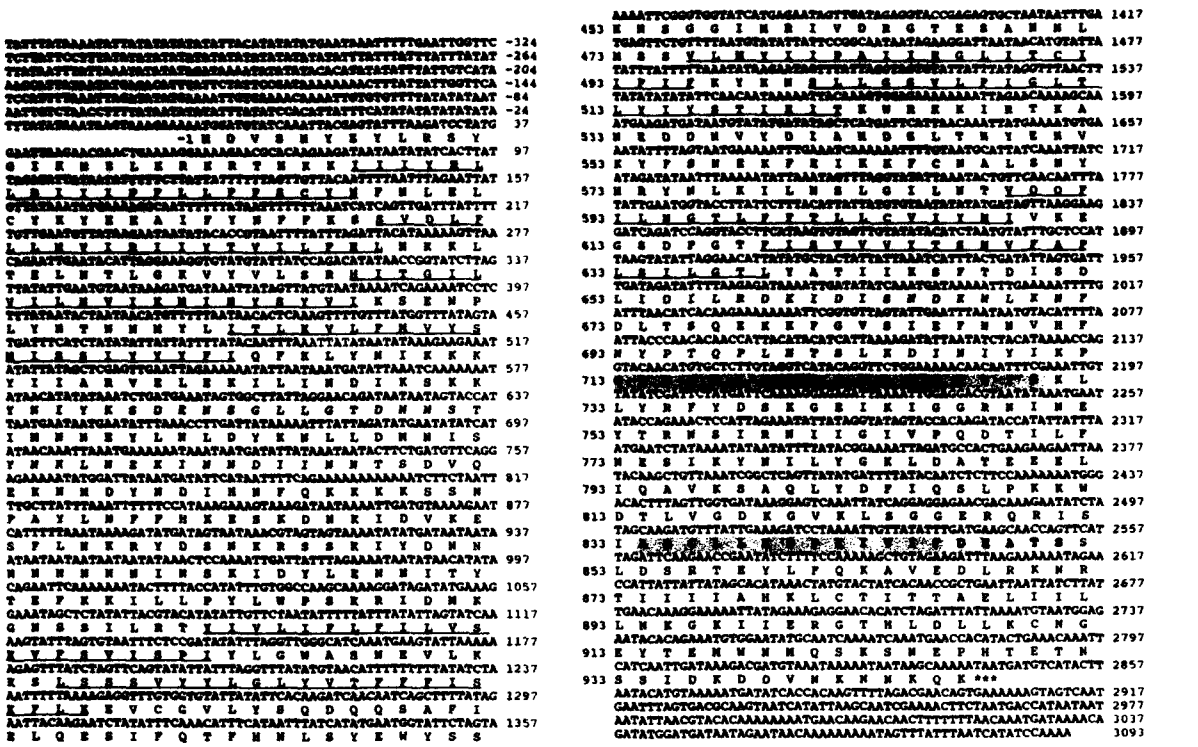

Fig. 4. Nucleotide sequence and the putative amino acid sequence of the *pfmdr2* gene. Nucleotides are numbered positively in the 5′-to-3′ direction, starting with residue 1 of the first in-frame ATG codon and ending with the last residue of the insert corresponding to the *Eco*RI cloning site of the clone λC1. Nucleotides in the 5′ untranslated region are numbered negatively beginning immediately 5′ to the first in-frame ATG codon and terminating at the first residue downstream at the *Eco*RI cloning site from the clone λP1C1. The deduced amino acid sequence of the *pfmdr2* protein is shown immediately below the nucleotide sequence and numbered at the left side of the sequence. The star indicates the position of the termination codon at the beginning of the 3′ untranslated region. The shaded areas represent the nucleotide binding regions. The underlined regions correspond to the transmembrane domains as predicted by the method of Eisenberg et al. [19]. The potential *N*-linked glycosylation site Asn-X-Ser is in the amino acid residue 138.

philic domain.

Sequence comparison. A search of protein sequence data banks with the *pfmdr2* amino acid sequence indicated that a carboxyl terminal segment containing the ATP-binding cassette displays sequence similarity with mdr-like proteins. In almost all cases, the amino acid sequence identity of these proteins with *pfmdr2* is confined to a 250 amino acid region surrounding the putative ATP binding site. The P-glycoproteins encoded by the human *mdr3* [29], human *mdr1* [30] and mouse *mdr2* [31] genes exhibit the highest level of sequence homology in that region with the *pfmdr2*. A comparison of the membrane spanning domains of the *pfmdr2* predicted protein with the available GenBank sequences identified of a protein *hmt1* encoded by the fission yeast *Schizosaccharomyces pombe* [8] which has a significant homology with the *pfmdr2* predicted protein. A pairwise alignment of the *pfmdr2* (947 aa residues) and *hmt1* (837 aa residues) has indicated that approximately 33% of all residues of *hmt1* can be matched with identical residues of *pfmdr2* and another 28% match with functionally similar residues. The secondary structure of both proteins was similar based on the 10 predicted transmembrane segments [19] and the predicted single ATP binding site (Fig. 6). *hmt1* is involved in heavy metal tolerance in yeast [8]. By sequence

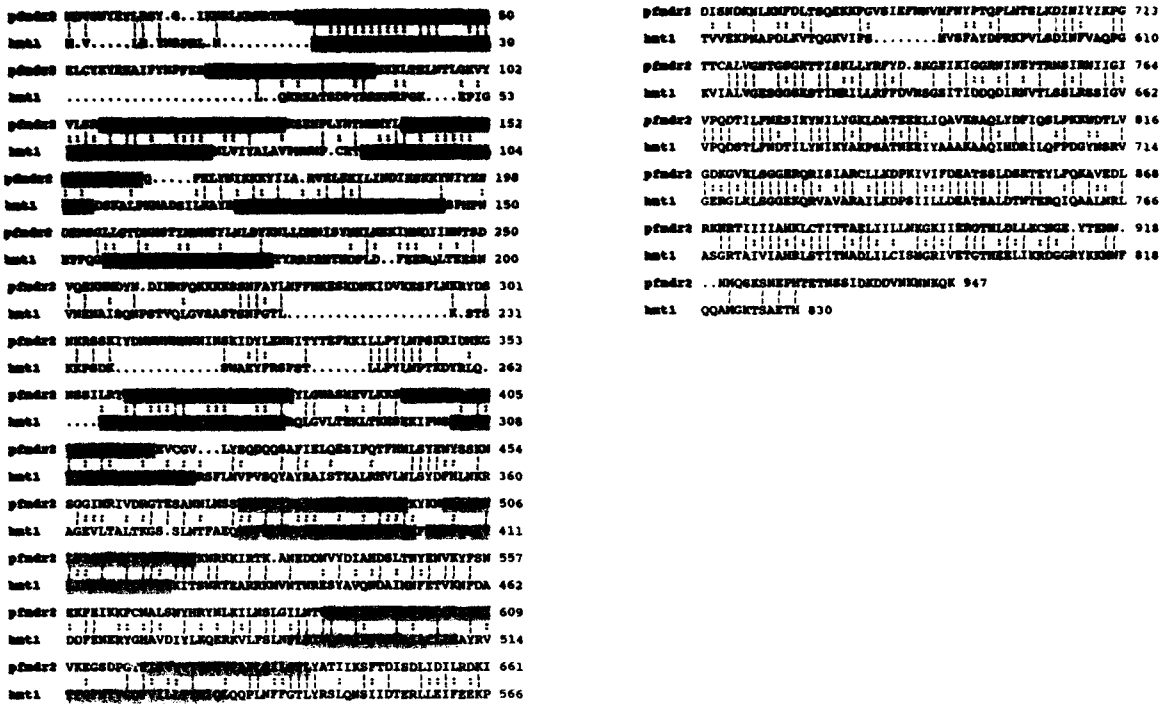

Fig. 5. Sequence comparison of the predicted *pfmdr2* protein and the yeast *hmt1* protein. The deduced amino acid sequence of the *pfmdr2* protein was aligned with the predicted yeast *hmt1* protein which participates in the transport of heavy metals. Dashes indicate gaps introduced in the sequence to produced optimum alignment. The bars (|) indicate identical residues and colons (:) indicate similar residues between the two proteins. The gray boxes in each sequence correspond to the putative transmembrane domains predicted by the algorithm of Eisenberg et al. [19]. Each sequence is numbered to the right of each lane starting at the first deduced Met residue.

and structure homology, these two protein can possibly share a role in heavy metal disintoxication in the cell.

Identification of the pfmdr2 protein. The *pfmdr2* protein was identified with antibodies raised to a fragment of the *pfmdr2* gene product expressed in *E. coli*. This construct produced a fusion protein which was purified and used to immunize rabbits (see Materials and Methods). The total rabbit anti-*pfmdr2* sera were used to identify and localize the protein by immunoblots and immunofluorescence (Fig. 7). The immunoblot analysis of *P. falciparum* W2 strain showed that the immune sera recognize a specific band of 110 kDa, that was not found when probed with the pre-immune sera. This is in agreement with the molecular weight that was predicted from the nucleotide sequence of *pfmdr2*.

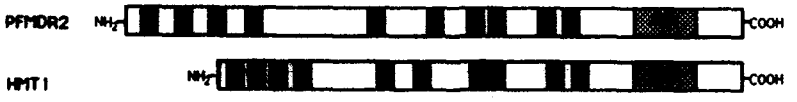

Fig. 6. Schematic representation of the *pfmdr2* and *hmt1* gene products. The gray box corresponds to the ATP-binding cassette at the carboxyl terminal region of the two proteins. The black boxes correspond to region of the putative hydrophobic membrane spanning domains of each protein, predicted by the algorithm of Eisenberg et al. [19]. Ten transmembrane domains were predicted for both *pfmdr2* and *hmt1* proteins. The longest ORF predicted 947 amino acids for the *pfmdr2* protein and 830 amino acids for the *hmt1* protein [8].

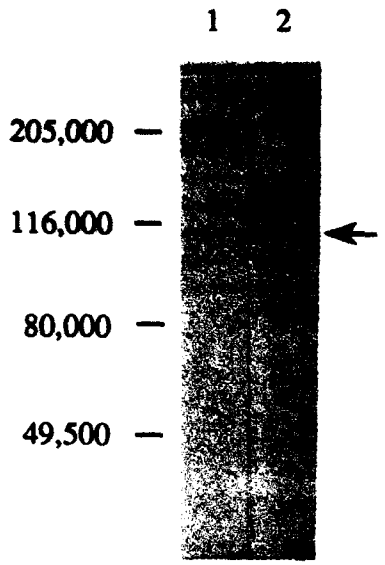

Fig. 7. Anti-*pfmdr2* antiserum identifies a single band of 110 kD (arrow) on western blot. Protein from W2mef saponin-lysed parasites (1.2 mg/lane) was separated in 8% SDS-PAGE and transferred by electroelution to nitrocellulose. The filters were reacted with: (*1*) its pre-immune serum at dilutions of 1:200, and (*2*) a rabbit polyclonal antiserum against a *pfmdr2* fusion protein (see Materials and Methods) and visualized with a second goat anti-rabbit IgG antibody linked to gold as described in Materials and Methods.

Asynchronous cultures of the HB3, W2 and W2mef strains were fixed to glass slides and examine by indirect immunofluorescence. The staining was limited to trophozoite and late stage parasites; no reaction with ring stage parasites was observed. The fluorescence occurred in a punctate pattern inside the parasite and was similar in the 3 strains analyzed. No reaction was found with uninfected erythrocytes (Fig. 8).

Discussion

The *pfmdr2* gene encodes a protein with 10 predicted transmembrane domains and a single ATP-binding site at the 3′ end of the gene. In a homology search using GenBank sequences, we discovered that the *pfmdr2* gene has a significant homology with the *hmt1* gene in yeast. The yeast *hmt1* gene is involved in cadmium resistance and is hypothesized to transport cadmium containing complexes. The predicted structure of the *hmt1* gene is similar to that of *pfmdr2*, namely 10 transmembrane domains and a single ATP binding site. We have further characterized the *pfmdr2* gene expression by northern analysis and discovered that it is expressed in a stage-specific manner, only at the trophozoite stage and not in ring stages. We have prepared a rabbit antibody to a recombinant fusion protein expressing a portion of the *pfmdr2* coding region. In IFA analysis by fluorescence microscopy, this antibody stains trophozoites and not ring stages and the antibody staining appears to have a vacuolar localization. Western analysis reveals a protein of approximately 110 kDa which is consistent with the size of the predicted open reading frame based on DNA sequence analysis. Based on this analysis and previous work, there is no evidence for a change in the *pfmdr2* expression or gene copy in drug-resistant versus drug-sensitive parasites. However, based the similarity with the *hmt1* gene of yeast, we plan to investigate the role of this gene in heavy metal resistance in the parasite.

A major question which remains is the role of this gene and its encoded protein in the normal parasite life cycle. The gene is expressed in a stage-specific manner in the erythrocytic portion of the life cycle. No information is available on its expression in the insect stages of the life cycle or in the liver and these remain as questions to be investigated in the future. One possible role for *pfmdr2* is in the transport of parasite specific molecules. Alternatively, the *pfmdr2* gene product may be involved in the export of toxic compounds.

Acknowledgements

This work was supported by grants from the NIH AI27872 and U.S. Army DAMD 17–91-C-1012. Mariano Zalis received support from the Tropical Disease Research Program of WHO/UNDP/World Bank. We thank Ramona Gonski for help in manuscript preparation.

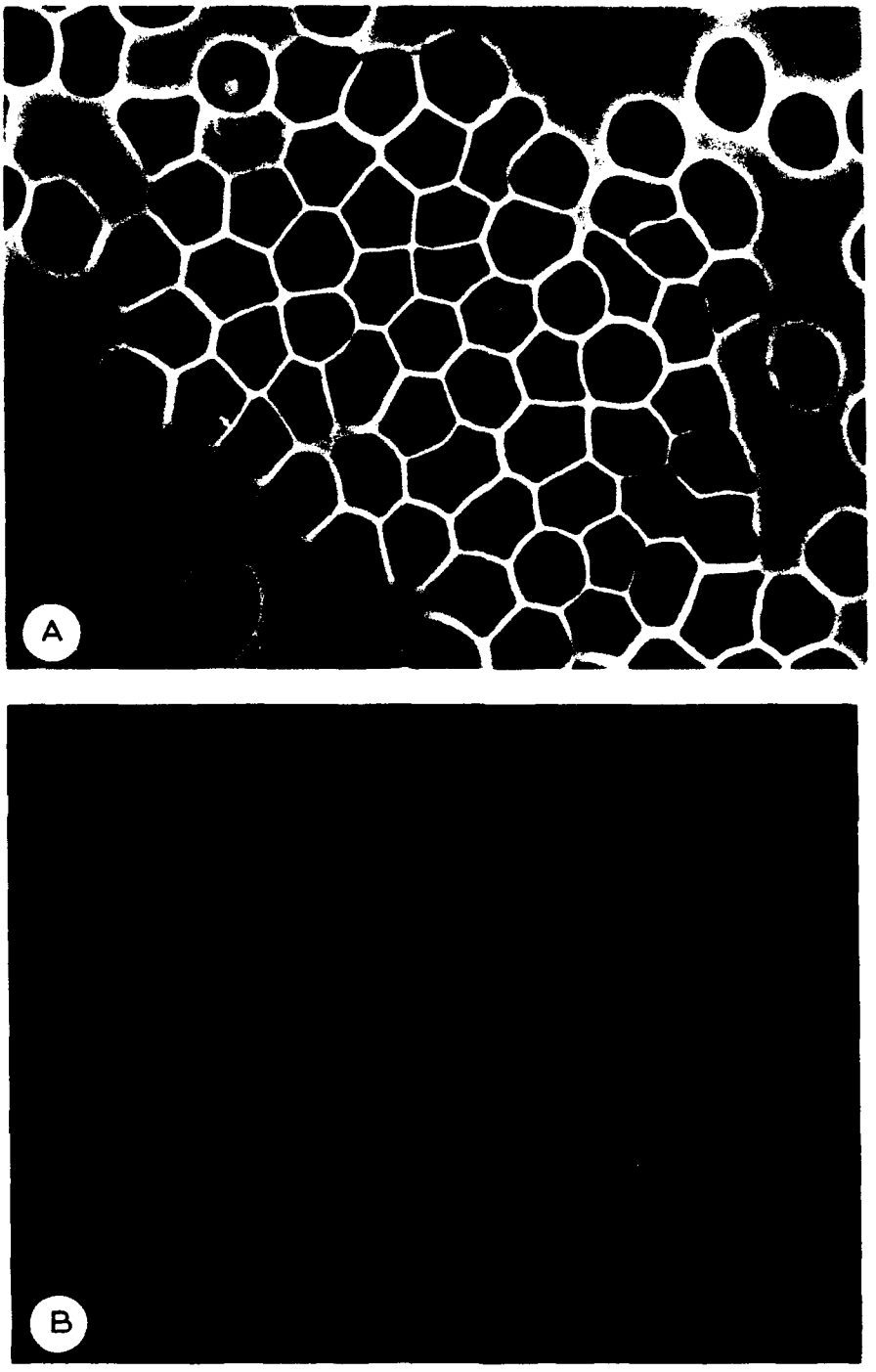

Fig. 8. Indirect immunofluorescence of W2 infected erythrocytes with anti-*pfmdr2* fusion protein. The slides containing fixed parasites were prepared from asynchronized W2 cultures as described in Materials and Methods. The cells were fixed with acetone and reacted with a rabbit anti-*pfmdr2* fusion protein (dilution 1 50) followed by FITC-conjugated goat anti-rabbit IgG. (A) Light Microscopy. The arrow indicates a ring stage parasite. The trophozoite forms of infected erythrocytes can be identified by the hemozoin granules (dark crystals) within the digestive vacuole. (B) Fluorescent micrograph. The anti-*pfmdr2* antibody only reacted with the trophozoite stage.

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Printed in The Netherlands

Reprint Series
9 June 1989, Volume 244, pp. 1184–1186

SCIENCE

Amplification of a Gene Related to Mammalian mdr Genes in Drug-Resistant *Plasmodium falciparum*

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The malaria parasite *Plasmodium falciparum* contains at least two genes related to the mammalian multiple drug resistance genes, and at least one of the *P. falciparum* genes is expressed at a higher level and is present in higher copy number in a strain that is resistant to multiple drugs than in a strain that is sensitive to the drugs.

HUMAN MALARIA IS WIDESPREAD throughout the tropical world and is a major human health problem in both morbidity and mortality. Infection with drug-resistant strains of *Plasmodium falciparum* is an evolving public health problem in nearly all endemic areas (*1*). Parasites resistant to high levels of chloroquine have necessitated the use of less effective and more expensive antimalarial drugs, but the parasites are becoming resistant to these other antimalarial drugs also. Neither the mechanism of action of the aminoquinolines nor the mechanism of resistance to these compounds is completely understood (*2*). Resistance of *P. falciparum* to chloroquine is associated with increased drug efflux (*3*), and this efflux can be reversed by verapamil and other compounds (*4*). Resistance to mefloquine can be reversed by penfluridol (*5*).

The finding that drug efflux and drug resistance can be reversed by verapamil is similar to findings in studies of drug resistance in mammalian tumor cells (*6*). The mechanism of resistance in tumor cells has been defined as increased drug efflux that is mediated by increased expression of the P-glycoprotein (*7*). This drug efflux can be inhibited by simultaneous administration of verapamil and related compounds (*8*). The sequence similarity of P-glycoprotein to bacterial transport proteins has led to the model that the P-glycoprotein is an adenosine triphosphate (ATP)–dependent efflux pump responsible for decreasing drug accumulation in resistant cells (*8*). The P-glycoprotein is encoded by the multiple drug resistance gene (mdr gene), which is amplified either at the DNA level or RNA expression level in tumor lines resistant to multiple drugs.

Thus one mechanism of drug resistance in *P. falciparum* may be similar to multidrug resistance in mammalian cells, namely the mediation of drug efflux by an ATP-dependent efflux pump (*3*, *4*). We tested this model by using sequences that are conserved in the mammalian P-glycoproteins and several bacterial transport proteins (*9*, *10*) to identify putative mdr-like genes in *P. falciparum*. Two primers based on conserved protein sequences shared in the mouse mdr, human mdr, and the bacterial hemolysin B(HlyB) proteins (*9*, *10*) were synthesized. The sense primer was based on a nine–amino acid homology found in position 1066–1075 of the murine mdr gene, and the antisense primer was based on a seven–amino acid homology found in position 1198–1204 (*10*). The codon usage was based on the preferred codon usage for *P. falciparum* (*11*).

These primers were incubated with DNA extracted from the W2 strain of *P. falciparum* under conditions of the polymerase chain reaction (*12*). Only those reactions that contained primers, enzyme, and *P. falciparum* template DNA showed the presence of an amplified sequence. The mdr gene primers did not amplify a sequence in human DNA. This is the predicted result, because human codon usage is different from that of the parasite, and the sequences chosen as primers would not be able to prime the human sequence. In using the mdr primers, there was an additional amplified band approximately 600 bp long and some other minor bands ranging in size from 300 to 1000 bp. The two major bands, approximately 450 and 600 bp in length, were used for further analysis. The identity of the other bands remains to be determined.

The sequence of the 600-bp (Pfmdr1) and 450-bp (Pfmdr2) fragments and the predicted amino acid sequences were compared with the sequence of murine mdr gene

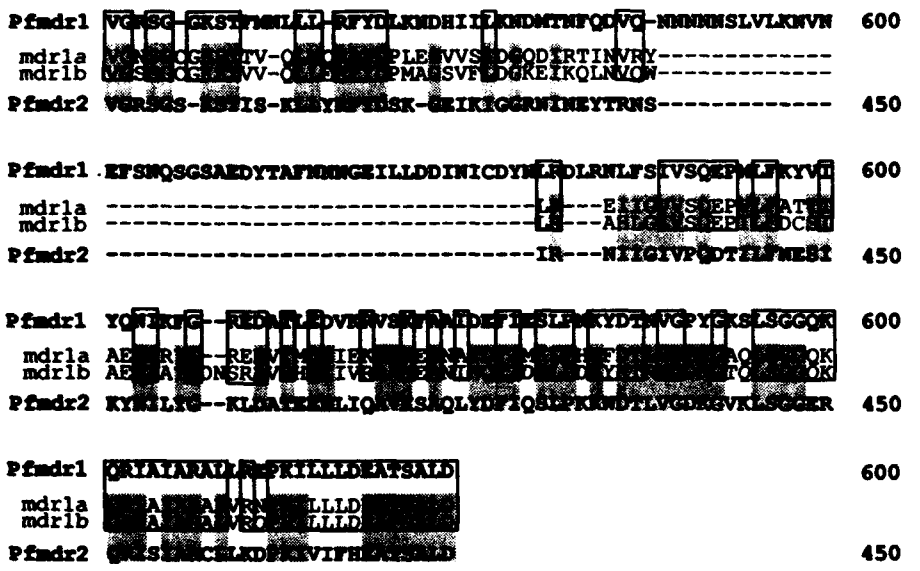

Fig. 1. Identification of mdr-like genes in *Plasmodium falciparum*. Comparison of the deduced amino acid sequence of Pfmdr1 and Pfmdr2 to the deduced amino acid sequence of murine mdr gene (*9*); mdr1a represents residues amino acids 425 to 562 and mdr1b represents residues 1068 to 1207 (*9*). The boxed (Pfmdr1) and shaded (Pfmdr2) areas indicate sequence identity between the *P. falciparum* genes and at least one of the two halves of the murine mdr gene. The selection of primers is discussed in the text. The two oligonucleotides synthesized (Biosearch 8750) with degeneracies in parentheses are (5′ to 3′) for sense strands, GT(A,T)-GG(A,T)-(C,A)G(T,A)-TC(T,A)-GG(T,A)-GG(T,A)-AAA-TC(T,A)-AC (degree of degeneracy, 256), and for the antisense strand, ATC-TAA-(T,A)GC-(T,A)GA-(T,A)GT-(T,A)GC-TTC-AT (degree of degeneracy, 16). Two additional primers containing synthetic Eco RI (sense) and Hind III (antisense) restriction sites were used in the cloning of the Pfmdr1 gene. The total polymerase chain reaction was extracted with phenol-chloroform and either cloned directly (Pfmdr2) or after restriction digestion (Pfmdr1) into the pBluescript vector at appropriate restriction sites (Eco RV for Pfmdr2 and Eco RI–Hind III for Pfmdr1). Transformants containing the putative mdr genes were selected by hybridization with radiolabeled polymerase chain reaction products, which were extracted from low melt agarose gels. Plasmids containing putative mdr genes were sequenced by using a modification of the dideoxynucleotide chain termination method with Sequenase enzyme (*16*). The data presented represents sequences of three independent clones for each gene. GenBank accession number for the nucleotide sequences of Pfmdr1 (600 bp) and Pfmdr2 (450 bp) are M24850 and M24851, respectively.

Department of Tropical Public Health, Harvard School of Public Health, Boston, MA 02115.

*To whom correspondence should be addressed.

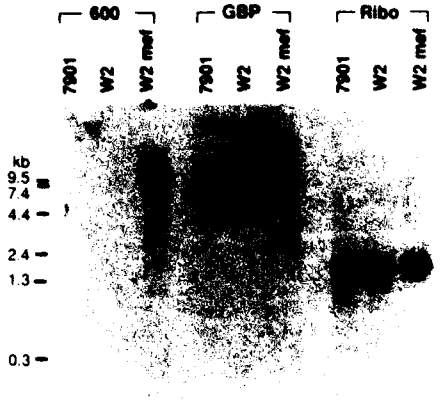

Fig. 2. RNA hybridization analysis with Pfmdr1. RNA was extracted from W2, W2 mef, and 7901. The median inhibitory concentrations to chloroquine are 40 to 60 ng/ml for W2, 20 to 30 ng/ml for W2 mef, and 3 to 5 ng/ml for 7901; and to mefloquine are 2 to 3 ng/ml for W2, 8 to 9 ng/ml for W2 mef, and 1 to 3 ng/ml for 7901. Cultivated parasites were washed and resuspended in RPMI supplemented with 30% human plasma. This suspension was layered onto a 75% to 76% Percoll gradient and centrifuged at 1200*g* for 30 min. The middle layers of concentrated parasitized red blood cells were washed twice in RPMI and suspended in a lysis buffer (50 m*M* Hepes, 2 m*M* EDTA, and 100 m*M* NaCl). Sarcosyl (10%) was added to a final concentration of 1% followed by vigorous mixing. The aqueous phase was repeatedly extracted with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1) until there was no interface material. The nucleic acid was precipitated with ethanol after the addition of sodium acetate (*p*H 5.2) to 0.3*M*. Equal amounts of total nucleic acid (10 μg) were resolved in triplicate on a 1.3% agarose gel in 0.66*M* formaldehyde. The RNA was transferred to nitrocellulose membranes, and hybridization was performed with three different probe molecules, Pfmdr1, the glycophorin binding protein (GBP) cDNA (*17*), and an rDNA probe. Autoradiograms of the RNA blots are shown after hybridization.

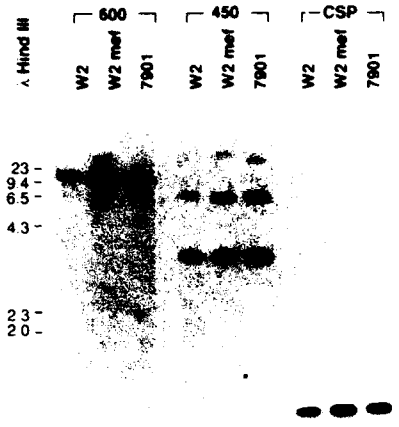

Fig. 3. DNA hybridization analysis of Pfmdr1 and Pfmdr2. DNA was extracted from strains W2, W2 mef, and 7901. Eco RI–digested DNA (1 μg) from each strain was resolved in triplicate on a 1% agarose gel and transferred to nitrocellulose membranes. Hybridization was performed as in Fig. 4. Three probes were used: Pfmdr1 (600 fragment), Pfmdr2 (450 fragment), and the CSP gene (*15*). Autoradiograms of the DNA blots after hybridization are shown. Quantitative densitometry was performed with a densitometer and the data are expressed as a ratio of the 450 or 600 hybridizing bands to the band hybridizing to the CSP probe. For the 450 fragment these values are 1.31 for W2, 1.47 for W2 mef, and 1.37 for 7901; for the 600 fragment the values are 0.62 for W2, 2.28 for W2 mef, and 0.92 for 7901.

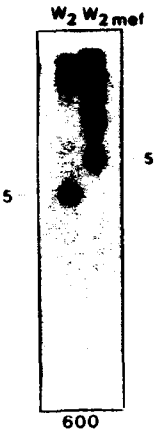

Fig. 4. Chromosome analysis of Pfmdr1. Agarose blocks containing *P. falciparum*–infected erythrocytes were prepared by standard techniques (*18, 19*). Separation of chromosomes was done at 150 mA (constant current), for 48 hours with a 2-min pulse interval at 16°C in a Beckman Geneline system. DNA was transferred to nitrocellulose membranes, and hybridization was performed according to the manufacturer's instructions. Chromosome 5 was confirmed by hybridization of parallel lanes with a known chromosome marker, MESA antigen (*18*).

(Fig. 1). The highlighted (boxed or shaded) regions indicate identical amino acids between the mouse mdr1 protein and Pfmdr1 (59% identity) or Pfmdr2 (58% identity). The Pfmdr1 fragment contains an additional 45 amino acids not in the mouse mdr1 gene or Pfmdr2. Pfmdr1 and Pfmdr2 are similar to each other (59% identity) at the amino acid level, but have little similarity at the nucleotide level. In the mammalian systems, there are also multiple mdr genes (three in rodents and two in humans) that are members of a closely related family (*13*), whereas in the *P. falciparum* system at least two different genes code for similar proteins.

If the mechanism of these putative mdr genes in *P. falciparum* is similar to that of mdr genes in human multidrug-resistant tumor cells, the model predicts that the gene would be expressed at a higher level in drug-resistant cells than in drug-sensitive cells. For this analysis, we used the cloned W2 and W2 mef lines of *P. falciparum*. W2 mef was derived from W2 by stepwise selection in increasing concentrations of mefloquine (*14*). RNA analysis reveals that in the W2 mef clone there is a significantly increased expression of an RNA molecule which hybridizes to Pfmdr1 (600 fragment) (Fig. 2). Equivalent amounts of RNA were analyzed from the W2, W2 mef, and 7901 strains, as demonstrated by the hybridization with probes specific for ribosomal RNA and the cloned glycophorin-binding protein cDNA. The Pfmdr1 transcript is longer than the glycophorin-binding protein transcript, which encodes a protein of 130 kD. This result is consistent with the Pfmdr1 gene encoding a protein of 150 to 170 kD. The use of RNA markers indicates that the size of the transcript is between 7 and 8 kb, which is larger than the 5-kb transcript of most mammalian mdr genes. The larger size of the transcript may be due to larger 5′ and 3′ untranslated regions in the *P. falciparum* mRNA. Longer exposure showed that the Pfmdr1 mRNA is also present in W2 and 7901 but at significantly reduced levels. This result indicates that the Pfmdr1 gene is expressed in both drug-sensitive and drug-resistant parasites.

In many of the mammalian tumor lines expressing high levels of mdr mRNA, there is also an amplification of the corresponding mdr gene. In DNA analysis with the same strains as used above, the W2 mef clone showed a two- to fourfold increase in the copy number of the Pfmdr1 gene when compared to its parent clone W2 and the 7901 strain (Fig. 3). There was no difference in the DNA copy number of Pfmdr2 (450 fragment) among the strains tested. Two other sensitive strains, 3D7 and Honduras, have a single copy of Pfmdr1. Quantitative densitometry analysis was performed with the single-copy circumsporozoite gene (CSP) as an internal standard (*15*).

The 600-bp Pfmdr1 sequence was also mapped by the pulsed-field gel technique (Fig. 4). In this case, two cloned parasites were compared, W2 and W2 mef. In W2, the 600-bp fragment maps to chromosome 5, whereas in the W2 mef strain it maps to a region containing several of the larger chromosomes of *P. falciparum*. Hybridization with a probe specific for another chromosome 5 marker (MESA antigen) indicated that chromosome 5 has increased in size, as expected with an internal amplification of the Pfmdr1 gene. In addition, as with the Southern blot, there is increased hybridization intensity in the W2 mef strain, again in agreement with this sequence being amplified.

The conclusion from these experiments is that *P. falciparum* contains at least two genes that are similar in sequence to the mammalian mdr genes and that at least one of these genes is expressed at a higher level and is present in higher copy number in one *P. falciparum* strain that is resistant to multiple drugs. These data are consistent with the

possibility that one mechanism of drug resistance in *P. falciparum* is similar to that of multidrug resistance in mammalian cells. Other mechanisms of drug resistance may also exist (*2*) and analyses of field isolates will be necessary to determine the importance of these observations to natural drug resistance in *P. falciparum*.

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20. We thank the members of the Chemotherapy of Malaria (TDR) steering committee for their early support and encouragement of this work and S. Aldritt and J. David for their careful criticism of the manuscript. Supported by a grant from the WHO/UNDP/World Bank Tropical Disease research program (to D.F.W.), a grant from John D. and Catherine T. MacArthur Foundation (to D.F.W.), and NIH training grants 1F23 AI07801-01 and 1K11 AI00892-01 (to C.M.W.). D.F.W. is a Burroughs-Wellcome scholar in molecular parasitology. A.W. and A.H.S. are students in the division of biological science at the Harvard School of Public Health. M.P.B. is a student at the Harvard Medical School.

7 November 1988; accepted 24 April 1989

Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 5234–5236, June 1993
Biochemistry

Transfection of the malaria parasite and expression of firefly luciferase

RENU GOONEWARDENE*†, JOHANNA DAILY*, DAVID KASLOW‡, TERRENCE J. SULLIVAN*, PATRICK DUFFY‡, RICHARD CARTER§, KAMINI MENDIS†, AND DYANN WIRTH*¶

*Department of Tropical Public Health, Harvard School of Public Health, Boston, MA 02115; †Malaria Research Unit, Department of Parasitology, University of Colombo, Colombo, Sri Lanka; ‡Malaria Section, National Institutes of Health, Bethesda, MD 20892; and §Institute of Animal Genetics, Edinburgh, EH93JN, Scotland

Communicated by William Trager, March 15, 1993 (received for review, February 24, 1993)

ABSTRACT **The goal of this work is to develop a method for the functional analysis of malaria genes using the method of DNA transfection. We have developed a transient transfection vector by constructing a chimeric gene in which the firefly luciferase gene was inserted in frame into the coding region of the pgs28 gene of *Plasmodium gallinaceum*. This plasmid DNA was introduced into *P. gallinaceum* gametes and fertilized zygotes by electroporation, and luciferase expression was assayed after 24 hr. This report of successful introduction and expression of a foreign gene in a malaria parasite demonstrates the feasibility of this approach to developing methods for the functional analysis of parasite genes.**

Malaria remains a major threat to world health. Efforts to control the disease have focused on chemotherapy, mosquito control, and, most recently, vaccine development. These efforts have been hampered by the emergence and spread of drug-resistant parasites, the breakdown of malaria control programs due to both insecticide-resistant mosquitoes and upheavals in spraying programs, and the complicated problems of vaccine development and testing. The world faces an increasing threat of malaria in the 1990s, with few new tools to combat the parasite or the disease (1).

One of the underlying problems in developing newer methods of control is that the basic biology of the parasite has not been fully investigated, primarily because of the lack of a method for functional analysis of genes and their products. The goal of the work presented here is to develop a method for the functional analysis of genes that uses the method of DNA transfection. This type of method is a critical next step in the functional analysis of parasite genes and is required for a detailed analysis of the control of expression of parasite genes. Such methods have been critical in dissecting the mechanisms of bacterial pathogenesis and, more recently, in the development of vaccines for several important bacterial pathogens.

The malaria parasite presents a unique challenge for transfection because it is intracellular for most of its life cycle. Introduced DNA must cross multiple membrane barriers before reaching the parasite nucleus. Because these multiple barriers would be likely to reduce the efficiency of introducing DNA into the parasite, we chose a parasite stage which is extracellular, the female gamete and fertilized zygote. Methods had previously been developed for the purification of gametes and fertilized zygotes of the avian malaria *Plasmodium gallinaceum* (2–5). Further, several genes of *P. gallinaceum* have been identified which are expressed at high level in the gamete/zygote stages of the parasite and one of these, pgs28, had been cloned with enough flanking DNA to assume that the necessary 5′ and 3′ controlling elements for the expression of this gene were available for developing a transfection vector (6).

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MATERIALS AND METHODS

Plasmid Construction. The pgs28 plasmid was previously described and the sequence is available in GenBank (accession no. M96886) (6). For these experiments, the pgs28 plasmid was digested with *Pst* I and *Sma* I to remove a small segment of the polylinker containing a *Bam*HI site, incubated with the Klenow fragment of DNA polymerase I under conditions to produce a blunt end at the *Pst* I site, and religated to create the pgs28.1 plasmid, which has a unique *Bam*HI site in the coding region of the pgs28 protein. The luciferase coding region (7) was obtained by PCR amplification of the pGem-*luc* (Promega) plasmid with primers [R1, 5′-GCGGATCCAGAAGACGCCAAAAACATAAAG-3′ (5′ end of gene), and R2, 5′-GCGGATCCAATTTGGACTTTC-CGCCCTT-3′] containing synthetic *Bam*HI sites. The *Bam*HI-digested PCR product was incubated with *Bam*HI-digested pgs28.1 under conditions for ligation. The resulting plasmid, pgs28.1LUC (Fig. 1), contains the firefly luciferase coding region inserted in frame with the pgs28 coding sequence. This structure was confirmed by restriction analysis, PCR analysis with primers 69a (5′-GGAAGCTTAACAGC-TATGACCATGATTAC-3′) and R2, and DNA sequence analysis. Plasmid pgs28.1LUCR was obtained by digestion of pgs28.1LUC with *Bam*HI and religation. The structure was confirmed by restriction digestion and PCR analysis as above. The pgs28.1GALR plasmid was obtained by a similar approach. The β-galactosidase coding region was purified from the pJ3-β-gal plasmid (gift of C. Cepko, Harvard University) after *Bam*HI digestion. The *Bam*HI-digested product was incubated with *Bam*HI-digested pgs28.1 under conditions for ligation and the resulting plasmid, pgs28.1GALR, has the β-galactosidase gene inserted in the inverse orientation relative to the pgs28 coding region.

Electroporation. White Leghorn chickens (3–5 weeks old) were inoculated with 0.1 ml of *P. gallinaceum*-infected blood and the parasitemia was followed daily. Blood was withdrawn (parasitemia 60–80%), and exflagellation and purification of fertilized zygotes and gametes were as previously described (2–5, 8, 9). The zygote/gamete fraction ($1–2 \times 10^7$ cells) was resuspended in Cytomix (10) and incubated in the presence or absence of plasmid DNA with or without electroporation. Unless otherwise indicated, all electroporations were performed under conditions of 2500 V/cm, 25 μF, with a time constant of 0.4. The cells were then washed and resuspended in ookinete maturation medium and incubated for 24 hr (11).

Luciferase Activity. The parasites were examined microscopically after 24 hr, pelleted, and suspended in luciferase

¶To whom reprint requests should be addressed.

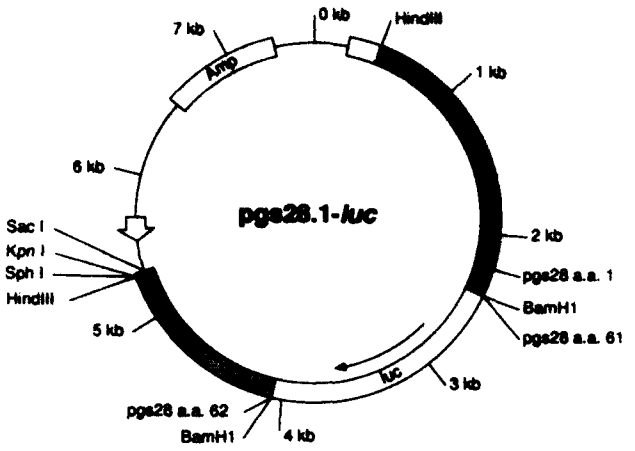

FIG. 1. Map of pgs28.1LUC. Amp, ampicillin-resistance gene; luc, luciferase gene; a.a., amino acid.

lysis buffer [25 mM Tris, pH 7.8 with H_3PO_4/2 mM 1,2-cyclohexanediaminetetraacetic acid/2 mM dithiothreitol/10% (vol/vol) glycerol/1% (vol/vol) Triton X-100]. In each experiment, an aliquot of the ookinete maturation medium was analyzed for sterility by plating in rich bacterial medium. No contamination was observed in any experiment. In each case, a standard curve was obtained at the same time for firefly luciferase (Sigma catalogue no. L5256, 18.9×10^6 light units/mg of protein) using the same substrate. The luciferase assay was performed with a luciferase assay kit (Promega, no. E1500) according to the manufacturer's instructions [substrates were 270 μM coenzyme A (lithium salt), 470 μM luciferin, and 530 μM ATP in 20 mM Tricine/1.07 mM $(MgCO_3)_4Mg(OH)_2$/2.67 mM $MgSO_4$/0.1 mM EDTA/33.3 mM dithiothreitol, final pH 7.8]. The results presented are in light units measured with an LKB BioOrbit 1251 luminometer. The amount of luciferase activity in each assay was calculated by using the standard curve which was assayed simultaneously. Each assay represents 10 μl of a 50-μl reaction mixture. For several experiments, an additional aliquot of each reaction mixture was assayed on a Lumat Berthold LB9501 luminometer.

RESULTS AND DISCUSSION

Two lines of experimentation were pursued simultaneously: the development of a system for the introduction of DNA into the gametes and zygotes and the construction of a transient expression vector which could be used to assay for expression of introduced DNA. Chickens were infected with *P. gallinaceum*, infected blood was withdrawn and allowed to undergo gametogenesis, and the gametes and fertilized zygotes were harvested and purified. These parasites were then suspended either in "suspended animation" (SA) buffer (10 mM Tris/170 mM NaCl/10 mM glucose, pH 7.4) or in Cytomix and subjected to electroporation under various conditions. The parasites were washed and incubated in ookinete development medium (11) for 24–48 hr. Ookinete development was then assayed by microscopic examination. Ookinete development was dependent both on the concentration of cells during development and on electroporation. The latter resulted in a lower yield of mature ookinetes. By using several different electroporation conditions and then assaying for ookinete development by microscopic examination, we determined electroporation conditions (2500 V/cm, 25 μF in Cytomix) which routinely gave an ≈50% reduction in the yield of mature ookinetes (data not shown).

The transient-transfection vector was constructed as follows. A 3.0-kb *Hin*dIII fragment including both the pgs28 coding region and flanking DNA was cloned into pUC13 and used for further development of the transient-transfection vector (6). DNA sequence analysis had identified a unique *Bam*HI site in the codons for amino acids 61 and 62 of pgs28 protein (6). Primers were prepared which allowed for the insertion of the firefly luciferase gene in frame at this position (plasmid pgs28.1LUC, see Fig. 1). Analysis of the resulting plasmid by PCR, restriction digestion, and sequence analysis has confirmed the in-frame insertion of the luciferase gene. A second plasmid (pgs28.1LUCR), in which the luciferase gene was inserted in the inverse orientation, was used as a negative control. A third plasmid (pgs28.1GALR), containing the bacterial β-galactosidase gene, was also used as a negative control.

Table 1. Expression of firefly luciferase in *P. gallinaceum*

Exp.	Cell no. $\times 10^{-7}$	Electroporation*	Plasmid (100 μg)	Luciferase, light units
1	2	+	pgs28.1LUC	154.7
	2	+	None	15.6
2	2.5	+	pgs28.1LUC	447.3
	2.5	−	pgs28.1LUC	7.9
3	1.3	+	pgs28.1LUC	179.2
	1.3	+	pgs28.1GALR	8.2
	1.3	+	None	8.8
4	1.1	+	pgs28.1LUC	707.3
	1.1	+	pgs28.1LUCR	8.5
5	1	1500 V/cm	pgs28.1LUC	86.3
	1	2500 V/cm	pgs28.1LUC	340.5
	1	3000 V/cm	pgs28.1LUC	21.5
6	0.75	+	pgs28.1LUC	746.5
	0.75	+	pGEM-LUC	8.8

*At 2500 V/cm, 25 μF unless otherwise noted.

All of the plasmids used for electroporation were purified by CsCl gradient centrifugation. Gametes/zygotes (2×10^7 cells) were electroporated in the presence of 100 μg of plasmid pgs28.1LUC. In control experiments, the same number of parasites were incubated in the presence of plasmid DNA without electroporation, electroporated in the absence of exogenous DNA, or electroporated in the presence of control plasmids (pgs28.1LUCR, pgs28.1GALR, pGEM-*luc*). The cells were washed and suspended in maturation medium. The cells were harvested after 24 hr and assayed for luciferase activity (Table 1). Luciferase activity was detected only in lysates of those parasites which had been electroporated in the presence of pgs28.1LUC. The amount of luciferase activity was dependent on electroporation conditions. No activity was detected in parasites incubated with that plasmid without electroporation or in parasites which had been electroporated with any of the control plasmids. In all experiments, an aliquot of each tube was assayed for sterility and no bacterial or fungal contamination was detected.

These results demonstrate transient expression of a reporter gene in the malaria parasite. The method constitutes the first step in the development of a transfection system. Clearly, the next step will be development of stable transfectants by using a selectable marker. Further, because the pgs28 gene is expressed only in sexual stages, modification of the flanking sequences to remove stage-specific controlling elements or identification of another gene expressed in other or all life-cycle stages will be necessary for analysis of gene expression in asexual or sporozoite stages. Nonetheless, the successful introduction and expression of a foreign gene in a malaria parasite demonstrate the feasibility of this approach to developing methods for the functional analysis of parasite genes.

K.M. was supported by the Fulbright International Scholars Program. We thank Dr. Peter David, Pasteur Institut, for initial discussions regarding gametes as targets for transfection. This work

was supported by grants from the National Institutes of Health (AI27872), the U.S. Army (DAMD 17-91-C-1012), and the John D. and Catherine T. MacArthur Foundation. J.D. is a Howard Hughes Medical Institute Physician Research Fellow. R.G. is funded by the World Health Organization Special Program for Research and Training in Tropical Diseases.

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